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MICROBIOLOGY

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CONTENTS

GROWTH STIMULATION OF MOLDS BY CARBON DIOXIDE AND EFFECT OF MESO-TARTARIC ACID ON THIS PROCESS

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We reached the conclusion, on the basis of previous experiments (Barinova, 1953) in which carbon dioxide was replaced by organic acids, that the stimulation by carbon dioxide of mold growth was not due to its participation in the synthesis of four-carbon dibasic and six-carbon tribasic acids, i.e., was not due to the participation of carbon dioxide in the Wood-Werkman reaction. In the present paper we have checked these conclusions in experiments with meso-tartaric acid which, according to studies of Quastel and Scholefield (1955), is a specific inhibitor of the Wood-Werkman reaction.

Quastel and Scholefield found that meso-tartaric acid in 0.02 molar concentration inhibits oxygen consumption and pyruvic acid oxidation by rat kidney cortex, but does not inhibit oxidation of citrate, α-ketoglutarate, and d,l-malate. The authors found further that the pyruvate oxidation inhibition produced by meso-tartaric acid is completely removed by addition of 0.002 molar fumaric acid, and partially removed by malic and citric acids. These observations led the authors to conclude that the action of meso-tartaric acid consists in inhibiting the formation from pyruvic acid and carbon dioxide of the oxaloacetate necessary to the functioning of the Krebs cycle (Braunshtein, 1957).

Since meso-tartaric acid, according to Quastel and Scholefield, inhibits the incorporation of carbon dioxide into oxaloacetic acid, then it will obviously remove the stimulating effect of carbon dioxide on mold growth if this stimulation is due to entry of the carbon dioxide into oxaloacetic acid synthesis; but if meso-tartaric acid exerts no effect on this process, the observed stimulation is consequently related to some other process.

METHODS

The experiments were done with the molds Aspergillus niger and Rhizopus nigricans. They were grown at 25-26 deg in submerged culture on a shaker in nutrient medium containing 1% glucose; .52% $NH_4H_2PO_4$; .042% K_2SO_4; .03% $MgSO_4 \cdot 7H_2O$; .003% $FeCl_3 \cdot 6H_2O$; .005% $ZnSO_4 \cdot 7H_2O$ and .003% $MnSO_4 \cdot 7H_2O$. In addition KH_2PO_4 (final concentration 0.05 M/liter) was added as buffer. To media not containing meso-tartaric acid, supplementary glucose was added in the same amount as the meso-tartaric acid. Air was passed over the nutrient medium at a rate of 10 liters/hour. Air entering cultures grown without carbon dioxide was carbon dioxide-free (Barinova, 1953). The mold was dried to constant weight at 80 deg. All experiments were conducted in triplicate in 250-ml flasks containing 30 ml nutrient medium.

In order to assure entry of the meso-tartaric acid into the cells, A. niger was grown at pH 3.1 and 3.8, and R. nigricans at pH 5.9 since it grows poorly at an acid reaction. Moreover, the meso-tartaric acid concentration in our experiments was twice as great (0.4 M/liter) as in those of Quastel and Scholefield.

Effect of Meso-tartaric Acid on Growth of Molds and on Stimulation of Growth by Carbon Dioxide.

It is seen from the experiment presented in the table that the action of meso-tartaric acid on growth of these molds depends on the cultural conditions. Meso-tartaric acid inhibits growth of A. niger and R. nigricans in cultures supplied with carbon dioxide-free air at all pH values tested; the more acid the nutrient medium is, the greater the growth inhibition due to meso-tartaric acid; thus, the inhibition of A. niger growth at pH 3.1 was 53.1%, while at pH 3.8 it was weaker and amounted to only 23.0%. Optimal reaction of the medium for R. nigricans is less acid than for A. niger, and it is probably for this reason that inhibition of R. nigricans growth was 31.8% even at pH 5.9.

Meso-tartaric acid has a toxic action on the molds grown under air containing the usual amount of carbon dioxide only at pH 3.1; inhibition of A. niger growth under these conditions is 24%, while at pH 3.8 meso-tartaric acid increases growth of A. niger by 22% and at pH 5.9 increases growth of R. nigricans by 12%, probably as a result of relative stabilization of the medium reaction. Consequently, the effect of meso-tartaric acid on molds depends on the reaction of the nutrient medium and on the presence of carbon dioxide in it. The relation between the effect of meso-tartaric on molds and carbon dioxide we will discuss in another paper.

As far the effect of carbon dioxide on mold growth, it is seen from the table that it stimulates the growth of A. niger and R. nigricans both in control cultures and in media containing meso-tartaric acid. Further, if a comparison is made between the action of carbon dioxide in absolute values and in relative values, then the relative stimulation of mold growth by carbon dioxide compared to control is 88-305.3% greater in cultures containing meso-tartaric acid. Consequently meso-tartaric acid in 0.04 M/liter amount does not inhibit stimulation of mold growth produced by carbon dioxide, but, in the contrary, increases this stimula-

Table 1. Effect of Meso-tartaric Acid on Growth of Aspergillus niger and Rhizopus nigricans and on Stimulation of Growth by Carbon Dioxide

Mold	Duration of growth (in hrs)	pH of medium before expt.	Meso-tartaric acid, M/liter	Air passed through: without carbon dioxide — pH after expt.	without carbon dioxide — mold wt. mg	without carbon dioxide — mold wt. in % of control	with carbon dioxide — pH after expt.	with carbon dioxide — mold wt. mg	with carbon dioxide — mold wt. in % of control	stimulation by carbon dioxide, %
Aspergillus niger	46	3.1	—	2.6	39.2	100.0	2.2	129.8	100.0	231.1
		3.1	0.04	2.9	18.4	46.9	2.6	98.7	76.0	536.4
Aspergillus niger	42	3.9	—	3.1	19.6	100.0	2.7	62.6	100.0	214.3
		3.8	0.04	3.6	15.1	77.0	3.2	76.4	122.0	405.9
Rhizopus nigricans	24	5.9	—	2.6	96.4	100.0	2.5	128.2	100.0	30.0
		5.9	0.04	4.6	65.8	68.2	3.4	143.6	112.0	118.2

tion. Carbon dioxide, absolutely, produces less mold growth on media containing meso-tartaric acid only at pH 3.0 inasmuch strong growth inhibition occurs, but at less acid reaction the mold growth is greater, absolutely, than in the control.

As has been pointed out, meso-tartaric acid is a specific inhibitor of oxaloacetic acid formation from carbon dioxide and pyruvic acid, according to the work of Quastel and Scholefield. Since in our experiments meso-tartaric acid does not inhibit mold stimulation by carbon dioxide, we can therefore conclude on the basis of the results obtained that the stimulation we observed of growth of these fungi by carbon dioxide is not due to participation of the carbon dioxide in synthesis of four-carbon and six-carbon acids.

Thus, the results of the study with meso-tartaric acid support our earlier conclusion.

SUMMARY

1. Meso-tartaric acid, 0.04 M/liter, inhibits growth of Aspergillus niger and Rhizopus nigricans in cultures supplied with air containing no carbon dioxide.

2. Meso-tartaric acid inhibits growth of A. niger in cultures supplied with air containing the usual amount of carbon dioxide only at pH 3.0, while at less acid reaction it slightly stimulates A. niger and R. nigricans growth, probably through relative stabilization of the reaction of the medium.

3. Carbon dioxide stimulates A. niger and R. nigricans growth both in the cultures containing meso-tartaric acid and in the control, the amount of stimulation being greater in the cultures with meso-tartaric acid.

4. Meso-tartaric acid does not remove the growth stimulation of A. niger and R. nigricans due to carbon dioxide.

5. Since meso-tartaric acid, a specific inhibitor of oxaloacetic acid formation from pyruvic acid and carbon dioxide, does not remove the stimulation due to carbon dioxide, this stimulation is consequently not due to entry of carbon dioxide into oxaloacetic acid synthesis.

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PARTICIPATION OF PROPIONIC ACID IN CHROMATIUM VINOSUM ANABOLISM

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The behavior of various groups of photosynthesizing bacteria toward organic compounds is the subject of investigation by scientists interested in determining the pathways of conversion of carbonic acid carbon to carbon of bacterial cell substance.

The reviews of Kondrat'eva (1954) and Elsden (1954), which thoroughly present the status of the problem over past years, make it possible to deal only with the latest work in this direction.

The assimilation of propionic acid carbon by photosynthesizing bacteria has now been shown by many workers. Thus, Kondrat'eva (1956) found that the purple non-sulfur bacterium, Rhodopseudomonas palustris, grows in the presence of propionic acid and another organic compound or CO_2. Studies were made by Maksimova (1957) with the same culture. Maksimova observed that with increased intensity of illumination the relative participation of propionate in photosynthesis decreases and the proportion of carbon dioxide in formation of cell substance increases, i.e. an alteration in the type of metabolism occurs in relation to light intensity: At low light intensities organic compounds are partially oxidized and partially assimilated directly; at high light intensities they mainly play the part of oxidizable substance. From this it follows that synthesis of cell substance from carbon dioxide reduction requires more energy than growth from photo assimilation of organic products. These data are in accord with the view of Shaposhnikov (1957), who believes that, since conversion of carbonic acid carbon to bacterial cell carbon proceeds through gradual build-up of organic compounds that do not accumulate in the medium in large amounts but are converted by the bacteria to more complex compounds, it is obvious that these intermediate compounds can be utilized more readily than CO_2 in anabolic metabolism.

Clayton (1957) concluded from a study of propionic acid metabolism in Rhodospirillum rubrum that propionic acid is utilized only if CO_2 is present in the medium, even in insignificant amounts, which is necessary to inititate the process of propionic acid conversion, whose first step is the carboxylation of propionic acid. Rejecting the mechanism proposed by Campbell and Stadtman of propionic acid conversion by oxidation to pyruvic acid through acrylic and lactic acids, Clayton considers more probable a carboxylation of propionic acid with formation of succinic acid, which then enters the tricarboxylic acid cycle.

Thus in Clayton's opinion CO_2 participates merely in the beginning of the pathway of propionic acid conversion; CO_2 addition to medium is not required later since adequate amounts can be formed in endogenous respiration. Since most studies have been made with members of the Athiorhodaceae, of particular interest are the studies of Nefelova (1955) with a typical representative of the purple sulfur bacteria, Chromatium vinosum. It was established for the first time by Nefelova that the purple sulfur bacteria too are able to utilize organic compounds in their metabolism. The Chromatium vinosum culture isolated by Nefelova grew on media containing organic acids, in particular propionic. Growth on propionic acid was observed only in the presence of CO_2 or other organic compounds. Here 0.5 mole of CO_2 was consumed per mole of propionic acid.

In comparing growth intensity of C. vinosum on media containing various organic compounds, Nefelova suggests that the possibility and intensity of utilizing organic compounds in the metabolism of purple sulfur bacteria depend on the structure of these compounds, extent of their state of reduction, presence or absence of a carboxyl group, and other properties. We showed in a previous paper (Shaposhnikov, Osnitskaya and Chudina, 1960) that C. vinosum can utilize acetic acid carbon in anabolic metabolism regardless of the presence of H_2S, CO_2, or other organic compounds in the medium, the acetic acid carbon consumed being completely or largely converted to cell carbon. We have here a case where, with respect to ration of the elements and with respect to state of reduction level, the acetic acid (or its aldehyde) and the organic substance formed during photosynthesis are approximately the same.

The process of utilizing the carbon of propionic acid in anabolism must be considerably more complex—a compound more highly reduced than bacterial cell substance on the average. Shaposhnikov (1957) believes that the participation in photosynthesis of propionic acid, being more highly reduced on the average than bacterial cell substance, must be associated with its oxidation which is carried out under anaerobic conditions only when hydrogen acceptors are present in the medium—carbonic acid or other carbon-containing compounds. However, the participation of propionic acid is not restricted to its oxidation; a direct incorporation of propionic acid carbon in the anabolism of purple bacteria is also indicated.

It was reported previously (Osnitskaya, 1958) that the C. vinosum culture we isolated can grow in the presence of organic compounds, certain of which in-

cluding propionic acid are utilized by the culture without adding as medium components other carbon sources or oxidizable compounds—hydrogen acceptors.

It was of definite interest to study in detail the dynamics of propionic acid consumption by purple sulfur bacteria and cell substance formation (with respect to carbon) under different cultural conditions, as well as the consumption in the presence of propionic acid of H_2S and CO_2 when these were added to the medium.

The work was conducted with our culture of C. vinosum. Experimental conditions and methods of determining cell carbon CO_2, SO_4, and H_2S have been described in a previous paper (Osnitskaya, 1958). Propionic acid was determined by the method of Sukhanovskii and Roginskaya (1936). Briefly, this method consists of the following: 50-100 ml of volatile acid distillate was oxidized with $KMnO_4$ (750 mg) in the presence of Na_2CO_3 (500 mg) with heating on a boiling water bath for 4 hours during which the propionic acid was oxidized to oxalic. After removing the MnO_2 precipitate with 96% alcohol, calcium oxalate was precipitated with a mixture of calcium acetate and glacial acetic acid. Oxalic acid was determined by the usual method. Experiments were conducted with Na propionate added in the amount 0.2% to van Niel medium from which, depending on conditions of the experiment, H_2S or CO_2 was excluded.

It was first of all necessary to determine to what extent propionic acid carbon is utilized to form cell substance during growth on propionic acid in the absence of other carbon sources, including CO_2. The inorganic van Niel medium without Na_2S or $NaHCO_3$ was so prepared that no CO_2 was present in it and precautions were taken to prevent its entry from outside. Determination of CO_2 in the medium at the beginning of the experiment indicated its absence. A special check was made of the purity of the propionic acid by qualitative reactions and Du Claux fractional distillation.

The data from these experiments are shown in Table 1.

As is plain from the numerical data in Table 1, C. vinosum utilizes the carbon of propionate acid. During 10-20 days the number of bacteria reaches 60-90 million cells/ml, biomass is 34-39 mg/100 ml. 2.5-5.8 mM propionate acid are consumed. Comparison of the amount of carbon contained in the bacteria with the amount of propionic acid carbon consumed shows that in most cases the propionic acid carbon is converted largely to bacterial cell substance and only an insignificant part remains in the medium in the form of intermediate or secondary products.

Formation of CO_2 in appreciable amounts in the medium was not observed. Thus these experiments warrant the assumption that propionic acid can serve as sole carbon source for C. vinosum.

Next, experiments were done to determine the possibility for propionic acid consumption on van Niel medium without $NaHCO_3$ but with Na_2S. Table 2 shows that under these conditions the culture grows very poorly: in 21 days the number of cells is 21 million/ml. Extremely insignificant also is the propionic acid consumption: in 21 days only 1.08 mM are consumed. Carbon of the acid consumed was utilized to form bacterial cells; no CO_2 was found in the medium.

It is seen from Table 3, containing data on propionic acid and CO_2 consumption during culture growth on van Niel medium containing $NaHCO_3$ but no Na_2S, that under these conditions too the amount of propionic acid decreases together with decrease in total amount of CO_2. In this case, however, there is a large discrepancy between the amount of carbon utilized from propionic acid and from $NaHCO_3$ and the amount of carbon found in bacterial cells; this is especially evident in experiment 6 where, obviously, a considerable part of the carbon remained in the medium in the form of intermediate or secondary products.

A similar picture is observed when the culture is grown on complete van Niel medium containing propionic acid (Table 4). Here also full correspondence between cell carbon and carbon consumed is not observed. This is perhaps a result of setting in of au-

Table 1. Propionic Acid Consumption by C. vinosum on van Niel Medium without Na S or NaHCO and Containing Propionic Acid

Expt. no	pH	Duration of expt., days	Cell harvest, million/ml	Biomass, mg/100 ml	Cell CO_2 found mM	Propionic acid consumed, mM
1	7.1	10	64.2	—	2.4	6.6
	7.2	18	65	—	4.4	13.2
2	7.4	9	62.7	—	3.3	8.6
	7.6	21	82.9	—	4.2	10.5
3	8.0	10	88.5	34	4.6	12.2
	8.1	12	90.3	39	5.8	13.6

Table 2. Propionic Acid and H S Consumption and Sulfate Formation in C. vinosum Grown on van Niel Medium without $NaHCO_3$

Expt. no.	Duration of expt., days	pH	Cell harvest, million/ml	Propionic acid consumed, mM	CO_2 in cells, mM	H_2S consumed, mM	H_2SO_4 formed, mM	H_2S utilized in mM per C atom
7	21	7.0	21.3	1.08	3	1.8	1.66	0.05
	30	6.9	23.8	1.08	3.8	2.4	2.2	0.04

Table 3. Propionic Acid and HCO_3^- Consumption on van Niel Medium with $NaHCO_3$ and Propionic Acid without Na_2S

Expt. no.	Duration of expt., days	pH	Cell harvest, million/ml	Biomass, mg/ml	Consumed in medium		CO_2 formed in cell, mM
					Propionic acid, mM	CO_2, mM	
2	9	7.5	25.5	—	2.4	0.9	8.0
	21	8.8	98.2	—	3.2	1.7	10.5
6	10	7.8	—	46	5.2	9.0	15.6
	15	8.3	—	48	7.5	12.9	19.9

Table 4. Propionic Acid, CO_2, and H_2S Consumption and Sulfate Formation on van Niel Medium Containing Propionic Acid + $NaHCO_3$ + Na_2S by C. vinosum

Expt. no.	Duration of expt., days	pH	Cell harvest, million/ml	Propionic acid consumed, mM	CO_2 consumed from medium, mM	Cell CO_2 formed, mM	H_2S consumed, mM	H_2SO_4 formed, mM	H_2S consumed in mM per
2	9	7.4	22.6	2.5	4.7	7.0	0.7	Traces	0.09
	21	7.15	50.0	4.7	4.5	12.0	0.87	Traces	0.06
1	10	8.1	93.3	4.0	1.5	12.2	2.9	1.1	0.19
	18	8.1	95.3	6.3	4.5	14.8	2.9	1.6	0.16

Table 5. Growth of C. vinosum on Propionic Acid with Decreased Amount of Seed Material (van Niel medium without $NaHCO_3$ or Na_2S and containing propionic acid)

No. of cells per ml medium at beginning of expt. (in 1000s)	Duration of expt., days	Biomass mg/100 ml	Propionic acid consumed, mM	CO_2 in medium, mM	Cell CO_2, mM
675	7	15	1.8	—	5.2
300	15	15	2.1	1.0	5.3
Less than 200	No growth in 1.5 months				

tolysis, and part of the carbon remains in the medium. It can be noted in examining the table that the relative propionic acid consumption is practically the same under various cultural conditions—with $NaHCO_3$ and Na_2S, with $NaHCO_3$, or without them. It is difficult to see a preferential consumption of propionic acid under any of these conditions. An exception is the case where Na_2S is present in the medium together with propionic acid, but there is no $NaHCO_3$. Here the consumption of propionic acid, as well as culture growth, is negligible. The hydrogen sulfide consumption in the presence of propionic acid is considerably less than on the usual van Niel medium with $NaHCO_3$ and without propionic acid.

It should be noted that growth of C. vinosum on propionic acid proceeds only when a considerable quantity of seed material is employed. Usually about 800 thousand per ml medium of seed material was employed in the experiments. Decrease to 300 per ml retarded the onset of growth; however, the culture later grew quite actively, consuming propionic acid and producing biomass (Table 5). When the amount of seed material was less than 200 thousand cells per ml, no growth was observed over a long period of time.

Thus, our culture of C. vinosum is able to utilize propionic acid in its anabolism during growth on inorganic van Niel medium to which, except propionic acid, no other additional organic acid or carbon dioxide was added.

At the same time, the fact that culture growth and propionic acid consumption are observed only when considerable amounts of seed material are inoculated and are absent when small amounts are seeded suggests that certain substances are introduced with the seed material that are able to initiate partial oxidation of the substrate.

In view of the absence of appreciable quantities of CO_2 in the experiments, one might assume that propionic acid conversion did not proceed via oxidation, but directly through participation of its carbon in forming cell substance. This, however, does not refute the scheme of Clayton, which explains most readily the metabolism of propionic acid. Further studies will help to solve this question.

SUMMARY

1. Chromatium vinosum can grow with propionic acid as sole carbon source under anaerobic conditions in the presence of light on inorganic van Niel medium without $NaHCO_3$ or Na_2S.

2. The amount of propionic acid carbon consumed has been shown to be almost equivalent to the bacterial cell carbon, indicating that this acid is utilized to form cell substance.

3. Propionic acid was consumed and CO_2 in the medium decreased on media containing $NaHCO_3$, or $NaHCO_3$ and Na_2S, in addition to the propionic acid. Complete correspondence between the amount of carbon consumed from propionic acid and $NaHCO_3$ and the bacterial cell carbon was, however, not observed. H_2S consumption was considerably less (0.09–0.15 mM

H_2S per carbon atom) than in cultures grown on conventional van Niel medium (0.5-0.7 mM H_2S per atom of carbon assimilated).

4. Culture growth and propionic acid consumption are very insignificant on medium containing propionic acid and Na_2S.

5. Culture growth is normal on medium containing propionic acid without $NaHCO_3$ only if a sufficient amount of inoculum is employed; the optimal number of cells is 500-800 thousand per ml medium. Reduction in amount of seed material to 300 thousand per ml medium results in greatly retarded growth, reduction to less than 200 thousand per ml medium results in no growth.

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CONTRIBUTIONS TO THE PHYSIOLOGY OF THIOBACILLUS THIOPARUS

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Thiobacillus thioparus is one of the principal organisms participating in the processes of hydrogen sulfide oxidation in nature.

Lees (1958) in his summary on the biochemistry of autotrophic bacteria refers Thiobacillus thioparus to the strict aerobes. However, studies of Al'tovskii, Kuznetsova and Shvets (1958) showed that this organism is frequently encountered in petroleum ground waters in which a considerable quantity of sulfides and hydrogen sulfide is present and there is no question of free oxygen. Similarly, a large number of thiobacteria were found by Lyalikova (1957) and Ivanov in the deep hydrogen-sulfide layers of water in Lake Belovod' (Kuznetsov, 1955) where, despite the fact that no dissolved oxygen was present, active chemosynthesis was observed.

The literature data on what sulfur compounds are oxidized by thiobacteria are also highly contradictory.

The purpose of the present paper was to isolate a pure culture of Thiobacillus thioparus and to study how the oxidation-reduction (redox) potential of the medium changes during culture growth and what sulfur compounds can serve as energy source for growth of this microorganism.

Isolation of a Pure Culture.

The starting material for isolation of a pure culture was a surface layer of silt from Lake Belovod'.

A mass culture was obtained by seeding this silt on Bejerinck's medium (Bejerinck, 1904). In 2-3 days the medium became turbid and on the surface a pellicle appeared consisting of, as shown by microscopic examination, amorphous sulfur formed during thiosulfate oxidation and masses of minute motile bacterial cells.

Seedings from an enriched culture, obtained from several successive transfers of pellicle to fresh portions of sterile medium, were made in dilutions on agar medium of the same composition. In 3-5 days minute colonies 1-1.5 mm in diameter appeared, white from the deposited sulfur.

To obtain a pure culture, colonies were seeded in liquid medium in flasks. In these transfers of individual colonies from solid medium to liquid, Thiobacillus thioparus did not grow as a rule, and if bacteria did grow they soon died upon subsequent transfers in liquid medium. In order to determine the reason for this, we employed Vishniac's medium (Vishniac and Santer, 1957) which differs from Bejerinck's in the assortment of trace elements, and we also added various vitamins and yeast autolyzate to the first medium. The addition of PABA (p-aminobenzoic acid), B_8 (i-inosite), B_6 (pyridoxine), nicotinic acid, and yeast autolyzate clearly improved growth appreciably, but even when they were present the pure cultures of Thiobacillus thioparus nevertheless died in 2–3 weeks.

Then we turned to the method of ZoBell (Hutton and ZoBell, 1949), employed by Kuznetsov and Telegina (1957) in isolating cultures of propane-oxidizing bacteria. It consists in making a mass seeding, 80-100 specimens, from colonies grown on solid medium into agar slant tubes and from these, liquid medium is seeded with a large amount of seed material and a check for purity is made simultaneously, making it possible to immediately detect and eliminate contaminated strains. We modified this method somewhat: to the agar slant tubes in which good growth of the seeded colonies was noted 2-3 ml of sterile liquid medium was added. By the next day a sulfur film appeared on the liquid surface, indicating good growth of bacteria in the liquid medium. The contents were transferred with a pipette to a flask containing liquid medium and a purity check was made at once by inoculating MPA and potato agar, since on these media the associated microorganisms grew well.

This method affords great economy of time and an opportunity to select immediately the active strains.

We succeeded in obtaining in this way several pure active strains, and one highly active one was kept for further study.

For a final check of purity a number of media were inoculated: MPA, MPB, MPB with glucose, wort with calcium carbonate, wort agar, potato agar, potato mince, potato slices, Vinogradskii's medium, Waksman's medium for Thiobacillus thiooxidans, Ballerud's medium for Thiobacillus denitrificans, and Bejerinck's medium without hyposulfite, with addition of 1% of various organic compounds: glucose, calcium lactate, sodium oxalate, acetate, or formate. The culture was considered to be pure if there was no growth on any of these media. Culture purity was checked by microscopic examination in addition to seeding on nutrient media.

The culture we isolated was identical in morphological and cultural characteristics to the Thiobacillus thioparus culture described by Bejerinck.

Relation of Thiobacillus thioparus Growth to Oxidation-Reduction Conditions in the Medium.

A study of the change in redox potential of the medium during Thiobacillus thioparus growth should to a certain extent describe the aeroboc or anaerobic

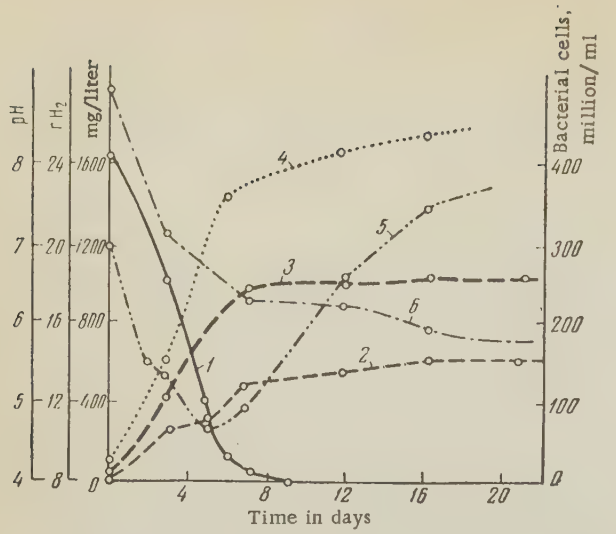

Fig. 1. Changes in oxidation-reduction potential during Thiobacillus thioparus growth on medium containing hyposulfite under aerobic conditions.
1) S/S_2O_3 ; 2) elementary S; 3) S/SO_4 ; 4) number of bacteria; 5) rH_2; 6) pH.

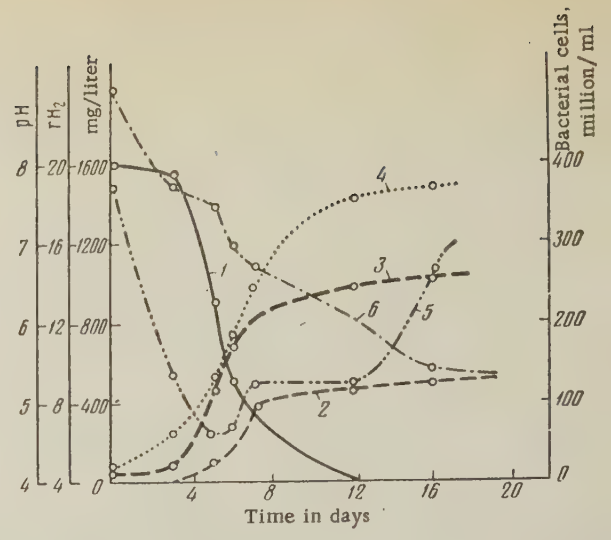

Fig. 2. Changes in oxidation-reduction potential during Thiobacillus thioparus growth on medium containing hyposulfite. Beginning of experiment under anaerobic conditions.
1) S/S_2O_3 ; 2) elementary S; 3) S/SO_4 ; 4) number of bacteria; 5) rH_2; 6) pH.

nature of this culture. At rH_2 of 15, virtually no dissolved oxygen in the medium is found by the method of Winkler.

Experiments were set up in vessels with a tube at the bottom; analytical samples were collected through the tube and an agar bridge was passed through the tube, necessary for contact between fluid and saturated potassium chloride solution in determining the oxidation-reduction potential of the medium. After sterilization the top of the vessel tube was closed with a rubber stopper in which was inserted a smooth platinum electrode for determining the oxidation-reduction potential and a glass stopcock with the use of which the experiment could be conducted in an atmosphere of any gases. The nutrient medium was inoculated with a pure culture of Thiobacillus thioparus, so that the initial bacterial concentration was about 20 million per ml.

Analytical data are shown in Figure 1.

As seen in the fiqure, growth of Thiobacillus thioparus proceeded most actively in the first five days and bacterial concentration reached 350 million per ml. The oxidation-reduction potential fell to 14 in the first two days and dropped to 10.3 on the fifth day. All the hyposulfite was oxidized on the eighth day to sulfur and sulfuric acid. Then bacterial multiplication proceeded more slowly and oxidation-reduction potential of the medium began to rise.

The experiments showed that in liquid medium the most active growth occurred when the oxidation-reduction potential did not exceed 12, i.e. when no dissolved oxygen was present in the nutrient medium.

The next experiment was set up to determine the lowest range of redox potential at which growth of this organism will still occur.

After inoculating the nutrient medium with Thiobacillus thioparus, the air was removed from the vessel and hydrogen. Later a slight amount of air was absorbed through the stopper, but for 12 days (to the end of bacterial growth) the oxidation-reduction potential of the medium did not rise over 9. As seen from Figure 2, under these conditions the bacteria multiplied, reaching on the twelfth day 350 million cells per ml, and virtually all the hyposulfite was oxidized to molecular sulfur and sulfuric acid.

Since the redox potential was greatly lowered during growth of Thiobacillus thioparus under stationary conditions, in order to study the effect of greater aeration in the surrounding medium on the oxidation of hyposulfite, experiments were set up in columns containing a tall layer of medium through which air could be passed at any rate.

Hyposulfite was added to the usual nutrient medium in the amount of about 3 g hyposulfite sulfur per liter. Air was passed through at a rate of 1, 10, and 60 volumes per hour per volume of medium in the column. Nutrient medium was inoculated with a pure active culture of Thiobacillus thioparus in the amount 20-30 million cells per ml.

The analytical data are shown in Figures 3 and 4. Where aeration rate was 1 volume of air per liter of medium per hour (Figure 3), the potential fell as the bacteria grew from 24 to 13. All oxidation of sulfur ended in 7 days. Where 10 volumes of air per hour per volume of liquid per hour was passed through, the potential did not fall below 19. Deposition of elementary sulfur began at the moment of fall in potential below 22.

Lastly, with strong aeration (60 volumes of air per volume of liquid per hour) the redox potential was 22 for a period of 7 days. As seen from Figure 4, almost no hyposulfite oxidation occurred and only on the 12th day, when the redox potential in the nutrient medium decreased to 16, did active oxidation of hyposulfite begin.

A duplicate experiment, except that the redox potential was not determined, showed approximately the same picture. Hyposulfite oxidation proceeded most

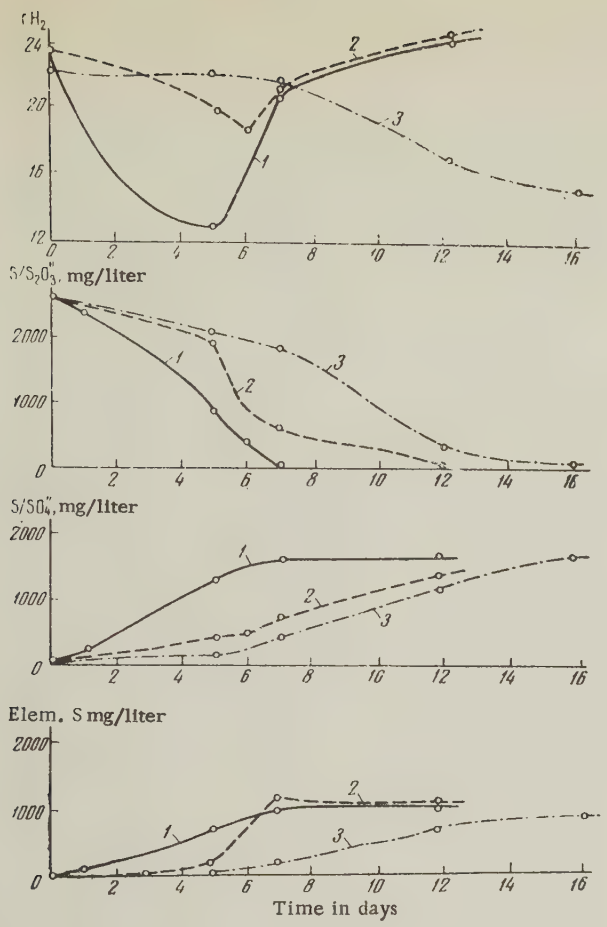

Fig. 3. Effect of degree of aeration of Thiobacillus thioparus on intensity of hyposulfite oxidation and value of oxidation-reduction potential in culture fluid.

Aeration rate: 1) 1 vol. air per hr per vol. nutrient medium; 2) 10 vol. air per hr; 3) 50 vol. air per hr.

actively under weak aeration and was virtually absent when the aeration rate was 60 volumes of air per hr per volume of liquid.

Oxidation of Various Sulfur Compounds by Thiobacillus thioparus.

As has been pointed out above, the question concerning oxidation of various sulfur compounds by thiobacillus remains obscure.

In view of this a number of experiments were set up to study the ability of Thiobacillus thioparus to oxidize different compounds of sulfur. Many of these are readily oxidized in the air and therefore controls were set up concurrently with experimental flasks, in which the thiobacteria were killed by addition of thymol or were not added at all.

No experiments were set up in many replicates in Erlenmeyer flasks to which 100 ml of inorganic nutrient medium was added.

The analytical results are shown below in a summary table.

It is seen from Table 1 that in control flasks there is almost no increase in amount of sulfates, except for sodium sulfite. The oxidation of this compound is chiefly chemical. Number of Thiobacillus thioparus cells increased during the 17 days of the experiment only 3-fold.

It was of much interest to determine the ability of Thiobacillus thioparus to oxidize sulfides. When sodium sulfide was present, the oxidation process proceeded slowly. Observations showed that at first the oxidation proceeds at approximately the same rate in control and experimental flasks with pH 7.6 of the nutrient medium, and on the 6th day a film of molecular sulfur of the nutrient medium. From this moment the thiobacteria in experimental flasks begin to oxidize the molecular sulfur and to acidify the nutrient medium.

The acidification of the medium in turn produces more active chemical oxidation of sodium sulfide to molecular sulfur, which is again oxidized by Thiobacillus thioparus.

Here also during the 17 days of experiment the number of bacteria in the culture increased only 3-fold. The analyses show that sulfide content in the experimental flask decreased by 312 mg/liter calculated as sulfur during this time. Since only about 45 mg/liter of sulfate sulfur was formed, it may be assumed that the bulk of sulfide volatilized from the medium as hydrogen sulfide.

The process of calcium sulfide oxidation proceeds entirely otherwise. Here 228 mg/liter sulfide calculated as sulfur was oxidized in 6 days in the experimental flasks. Of this, 154 mg was oxidized to sulfuric acid, no sulfate increase being noted in the control.

The oxidation of calcium sulfide by Thiobacillus thioparus in relation to the acidity of the medium is shown in Table 2.

As seen from Table 2, initial pH of nutrient medium was 6.9; 7.15; 7.32; 7.50; 7.85. The medium became

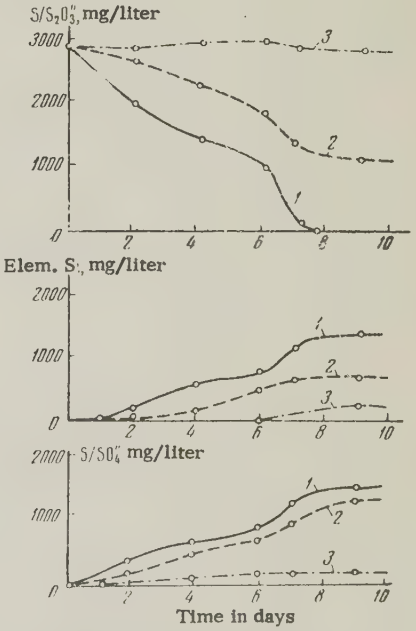

Fig. 4. Effect of degree of aeration of Thiobacillus thioparus on intensity of hyposulfite oxidation.

Aeration rate: 1) 1 vol. air per hr per vol. nutrient medium; 2) 10 vol. per hr; 3) 60 vol. per hr.

Table 1. Utilization of Various Sulfur Compounds by Thiobacillus thioparus (Bacteria in million/ml, compounds in mg/liter)

Sulfur compound	Duration of expt. in days	No. of bacteria			$S/S_2O_3^{=}$		$S/SO_3^{=}$		$S/S^{=}$		Selem.		$S/SO_4^{=}$	
		expt. variant	start	final	start	final	start	final	start	final	start	final	start	final
$Na_2S_2O_3$	6	expt.	20	439	1600	0	—	—	—	—	0	—	44	1032
		contr.	20	200	1600	1600	—	—	—	—	0	0	44	44
Na_2SO_3	17	expt.	95	331	0	0	208	32	0	0	—	—	726	902
		contr.	95	115	0	0	208	56	0	0	—	—	726	874
Na_2S	17	expt.	66	200	0	0	—	—	312	0	—	—	153.6	198,4
		contr.	49	49	0	0	—	—	312	148	—	—	153.6	162
CaS	6	expt.	49	207	0	0	—	—	228	0	+	—	73.6	227.2
		contr.	49	49	0	0	—	—	228	40	0	—	73.6	73.6
Elementary S	17	expt.	185	423	0	0	0	0	0	0	excess		32.0	294.4
		contr.	185	185	0	0	0	0	0	0			92.0	98.4

Table 2. Growth of Thiobacillus thioparus on Medium with Calcium Sulfate (Cells in million/ml, compounds in mg/liter)

Expt. variant	1/9 (seeded)			1/12				1/25			
	pH	S/CaS	$S/SO_4^{=}$	cells	pH	S/CaS	cells	pH	S/CaS	$S/SO_4^{=}$	cells
Expt.	7.85	416	67.2	34.8	8.25	352	74.1	6.80	0	476.8	331.8
Contr.	7.85	416	67.2	0	7.92	160	0	8.60	0	70.7	0
Expt.	7.50	416	67.2	34.8	8.05	280	78.1	6.90	0	412.8	300.1
Contr.	7.50	416	67.2	0	7.60	272	0	7.80	128	64.0	0
Expt.	7.32	416	67.2	34.8	7.83	32	185.2	6.60	0	457.4	253.0
Contr.	7.32	416	67.2	0	7.52	80	0	7.83	0	70.4	0
Expt.	7.15	416	67.2	34.8	7.40	80	146.3	6.70	0	496.0	225.1
Contr.	7.15	416	67.2	0	7.43	112	0	7.70	0	86.4	0
Expt.	6.90	416	67.2	34.8	7.37	48	165.7	6.70	0	496.0	184.9
Contr.	6.90	416	67.2	0	7.60	32	0	7.78	0	80.0	0

more alkaline during the first three days. Then acidification began. All sulfide was oxidized to sulfur and sulfuric acid in experimental flasks in a period of two weeks. The most active bacterial growth and sulfide oxidation occurred with initial pH of 7.3.

At the end of the experiment, regardless of initial pH, the acidity of the medium increased and pH varied in the range 6.6-6.9.

In control flasks at the end of the experiments sulfide was also absent, but amount of sulfate scarcely increased. Obviously part of it was oxidized by oxygen of the air to intermediate compounds, and part left the solution in the form of hydrogen sulfide.

Thus the experiments have shown that the Thiobacillus thioparus culture at our disposal is able to utilize for its multiplication the energy of oxidation of hyposulfite, elementary sulfur, calcium sulfide, and to a small extent sodium sulfide.

DISCUSSION OF RESULTS

The comparison of results from experiments on oxidation-reduction potential in a Thiobacillus thioparus culture indicates that this organism grows best at somewhat lowered values, i.e. that this organism is not a strict aerobe. The question remains obscure of how hyposulfite oxidation can proceed when rH_2 in the nutrient medium varies from 9 to 12.

It may be suggested that diffusion of atmospheric oxygen may have occurred and the redox potential in the layer of contact between nutrient medium and air was higher. Studies must next be made in this direction. The observations also showed that the contradictory data of different authors regarding the ability of Thiobacillus thioparus to utilize sulfides as an energy source depend on what salt of hydrogen sulfide was employed in the experiment.

SUMMARY

1. Thiobacillus thioparus grows best in medium with oxidation-reduction potential in the rH_2 range 10-16. Consequently this organism cannot be regarded as a strict aerobe.

2. Of the energy sources we studied, Thiobacillus thioparus can utilize hyposulfite, elementary sulfur, and calcium sulfide.

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EXPERIMENTAL VARIATION IN ASPERGILLUS NIGER

I. MORPHOLOGICAL CHARACTERISTICS OF VARIANTS OBTAINED BY ULTRAVIOLET IRRADIATION

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The sphere of practical application of various mold fungi becomes more extensive every year. Certain species of the aspergilli have been given particular attention from among the fungi, since they form a number of antibiotics and organic acids, as well as the enzymes protease, amylase, and pectinase. It is entirely natural that the activity of various aspergillus cultures is of much importance. Yet the progress in aspergillus selection is much more modest than the results of analogous studies on penicillium. From the latter, as is known, mutants were obtained by means of radiant energy that formed 100 times more penicillin than the original wild form of this fungus. Modern genetics of microorganisms, in solving a number of important theoretical questions, does not direct adequate attention to obtaining more active variants of microorganisms. The obtaining of active strains by means of radiant energy should not have a random character. As a result of extensive experimental work all the conditions should be determined under which, when they have been set up, it is possible to consistently reproduce previously obtained results and to isolate again with certainty mutants with specific physiological properties. In the process of investigation the relation between dose of radiant energy is established on the one hand, and occurrence frequency of mutations with the necessary properties, on the other. At the same time a determination is made of the degree of correlation between various morphological characteristics and biochemical activity. The investigator determines the possibility of obtaining a specific variant from a single treatment with radiant energy or concludes that repeated irradiation of the selected variant is necessary. The stability of the mutant obtained and its physiology is also of much interest, since the variants, in order to realize their biochemical potentialities, not infrequently require nutrient media of a composition entirely different from those on which the original strain was cultured.

What has been said does not, of course, give a full conception of the tasks confronting the investigator, but it well illustrates the indisputable principle that the systematic obtaining of mutants with specific properties is possible only after a determination of the conditions under which the mutants arise and a detailed study of the physiology of the altered forms.

The present investigations deal with the variation in an Aspergillus niger culture which forms citric acid. To determine the possibility of obtaining variants that form more citric acid than the original culture was the ultimate aim of the work.

METHODS

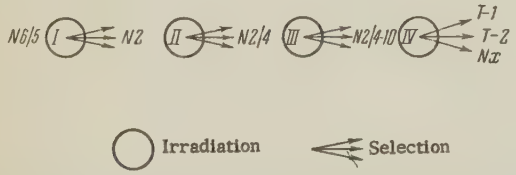
In choosing a culture, we selected the strain Aspergillus niger 6/5, since, being most active, it was employed at the Leningrad citric acid plant to obtaining citric acid by the surface method. First, several single-conidium cultures were isolated by the drop method and their ability to form citric acid was checked. Their activity was the same as the maternal strain and all further investigations were made with one of the single-conidium cultures.

In order to avoid possible variation of the culture under laboratory conditions, the conidia collected from the culture grown on wort agar containing 1.5% sodium chloride, were kept in sterile tubes in the refrigerator or in sterile dry soil also in tubes. In each experiment the conidia from soil were seeded on wort agar containing salt, since particularly active conidium formation is observed on this medium. After 4-day growth of the culture at 32 deg, the conidia were removed and suspended in sterile tap water. The water contained 7 million conidia per ml. To a standard Petri dish on a movable stage was added 4 ml of the suspension. Plates were irradiated with ultraviolet rays under a BUF lamp for 6 minutes 40 seconds (wave length 2537 A). During this time each conidium received $96 \cdot 10^{-3}$ erg. The need for such a dose was indicated by studies of Kuzyurina (1959) conducted in our laboratory which established the great stability of Aspergillus niger conidia toward ultraviolet rays. After irradiation the conidum suspension was diluted, the diltuions being various—from 1:200 to 1:2000. The diluted conidium suspension was seeded on Petri dishes containing wort agar. Inoculated plates were kept for 3-4 days at 32 deg; then the morphology of colonies grown on the plates was studied. From colonies whose morphology was different from that of the original strain, seedings were made into tubes containing wort agar. Next, the acid-forming capacity of all isolated strains was determined in cultures grown on sucrose-containing inorganic medium of the following composition: sucrose 5.0 g; NH_4Cl—0.35 g; $MgSO_4$—0.05 g; KH_2PO_4—0.05 g; $ZnSO_4 \cdot 7H_2O$—0.005 g; $FeSO_4$—0.0025 g; tap water—100 ml with replacement of this medium

after 2 days with 20% sucrose solution containing 0.19% ammonium chloride. Acidity was determined by titrating 1 ml of a 4-day culture on the second medium with 1/10 N NaOH. Cultures more active than the original strain were selected and studies further.

Obtaining Variants.

Among the colonies which grew out after seeding irradiated aspergillus conidia were some that differed in form and structure from colonies of the original strain. Seedings from these colonies gave cultures whose acid-forming capacity was determined. After checking 22 such cultures, attention was directed to cultures No. 1 and No. 2. They were irradiated a second time under the same conditions. Then again a selection was made and the activity checked. As a result culture 2/4 was selected and irradiated a third time. After seeding the irradiated conidia, a particular colony was selected from those that grew out, from which culture 2/4-10 was obtained. Lastly, this culture was irradiated a fourth time and after selection cultures T-1, T-2, and "X" were obtained. Thus, we employed repeated irradiation which alternated with selection and checking of activity. A conception of this multistep method of selection is given in the diagram.



The question of course arises of whether the repeated action of ultraviolet rays is absolutely necessary in order to obtain active mutants. We do not as yet have sufficient experimental data to decide this question conclusively.

Certain observations, however, made in the course of our work, indicate that the mutants obtained after one or two irradiations, although they possessed increased biochemical activity, were not as stable as those arising after four irradiations. In obtaining variants of penicillium with increased ability to produce penicillin (Imshenetskii, 1951), and variants of aspergillus that form active proteases (Brotskaya, 1956), it is likewise expedient to carry out repeated irradiation.

MORPHOLOGY OF VARIANTS

Form and structure of colonies and morphology of mycelium, conidiophores and conidia were studied in the variants obtained. The results of these investigations were compared with the structure of the original Asperillus niger 6/5 culture. We therefore give first of all a description of this culture.

Aspergillus niger 6/5, Original Culture.

A 5-day colony on wort agar is round, 6.5 cm in diameter, with flat even edges. The colony is flat with a rough surface and rather long aerial mycelium. Conidial heads are distributed all over the colony surface. Colony color is brownish black. The colony edge is in the form of a rather narrow white border (Figure 1). The bottom surface of the colony is whitish yellow and smooth, except for a slightly wrinkled center with

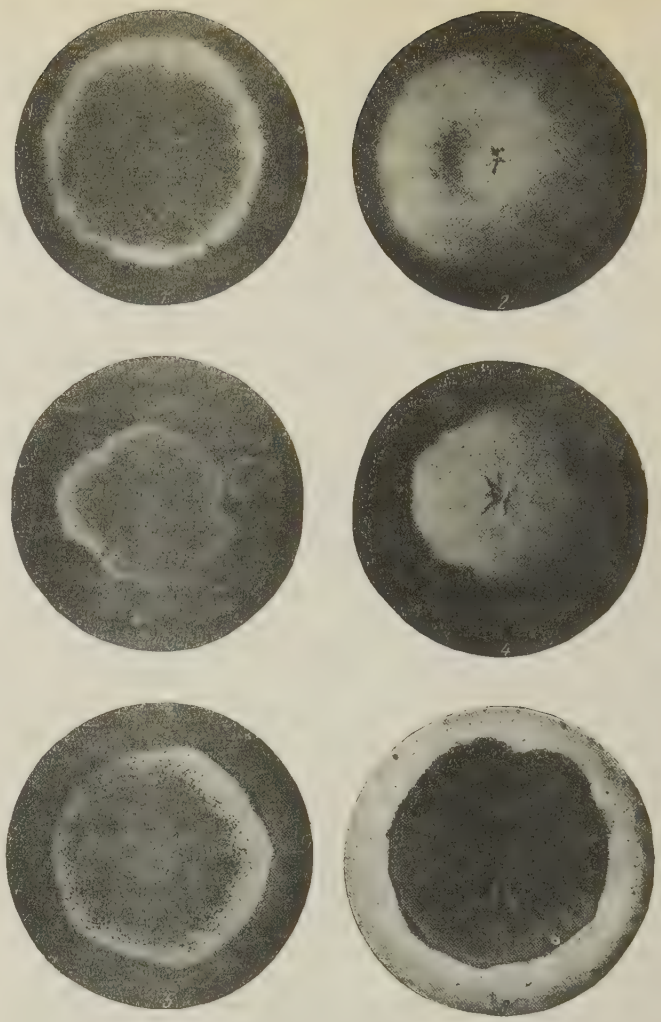


Fig. 1. Original A. niger 6/5 culture.
5-day colony, natural size, view from top.
Fig. 2. Same as Fig. 1, view from bottom.
Fig. 3. Variant 2/4-10.
5-day colony, natural size, view from top.
Fig. 4. Same as Fig. 3, view from bottom.
Fig. 5. Variant T-1.
5-day colony, natural size, view from top.
Fig. 6. Same as Fig. 5, view from bottom.

short radial folds about 0.8 cm in length (Figure 2). This factory culture has little resemblance to wild forms of Aspergillus niger isolated from natural sources: soil, food, etc. The latter form a large number of conidia all over the colony surface; the mycelium is always relatively fine and straight, and hence the wild strains form flat smooth colonies. In contrast to this, Aspergillus niger 6/5 resembles "cultivated" strains obtained experimentally with less abundant conidia formation, which is seen particularly well in cultures grown on wort agar slants in tubes. Here conidia are formed only in the upper third of the agar slant. Strain 6/5 conidia are somewhat polymorphous. Their diameter varies in the range 3.5-4.8 . Conidia 4.2 μ in diameter are encountered most frequently. Mycelial hyphae are quite thick, 3.5-6.8μ. The mycelium curves, producing a wrinkled type of growth particularly evident in liquid nutrient media. Cells composing the hyphae are relatively short, 35-110μ. Conidiophores are somewhat thicker than usual, 24-

Table 1. Dimensions of Colonies, Mycelial Hyphae, Conidiophores, and Conidia in Original Aspergillus niger 6/5 Culture and Mutants 2/4-10, T-1, T-2, and "X" Obtained by Ultraviolet Irradiation (5-Day Colonies on Wort Agar)

Culture	Giant colony diam. in cm	Thickness of mycelial hyphae in μ	Length of mycelial cells in μ	Thickness of conidiophores in μ	Diam. of heads in μ	Diam. of conidia in μ
6/5 (original)	6,5	3,5—6,8	35—110	24—27	67—99	3,5—4,8. чаще 4,2
Mutant 2/4-10	4,5—5,0	1,2—6,8		9,5—18,5	34—104	3,3—4,8. ген. 4,2
Mutant T-1	4,8—5,0	1,8—6,5	26—75	11,7—20,6	34—105	2,7—6,0. ген. 4,5
Mutant T-2	4,5	1,7—9,5	30—173	9,6—23,6	64—83	2,6—5,1. ген. 3,8
Mutant «X»	2,5	4,1—5,1	18—70	14,3	50—140	3,0—5,6. ген. 3,6

27μ. Size of conidial heads varies in the range 67-99 (Table 1).

Mutant 2/4-10.

Obtained after three irradiations, first of conidia of culture 6/5 and then of conidia of two selections of mutants. Size of 5-day giant colonies on wort agar reaches a diameter of 4.5-5.0 cm. Shape of colony is not always round and regular, sometimes it has a lobular border (Figure 3).

Colony surface is rough, with unequally distributed conidial heads which are arranged concentrically and form a darker circle at some distance from the colony center. Center and edge of the colony are lighter. Color of conidial heads is brownish black and is considerably darker than in the original 6/5 culture. Aerial mycelium is high. Bottom surface of the colony is smooth, except that the center has cleft-like depressions from which radial, convex folds proceed, reaching 1 cm in length (Figure 4). Colony diameter in the mutant is appreciably less than in the original culture (Table 1) and wrinkling is somewhat greater. There is also observed a certain decrease in minimal thickness of mycelial hyphae, 1.2-6.8μ, and of conidiophores, 9.5-18.5μ. Size of conidial heads is 34-104μ. Thus smaller heads are found in the mutant than in the original culture. But the size of conidia is the same as in the original culture, 3.3-4.8μ. Intensity of conidia formation is likewise unchanged.

Mutant T-1.

The culture was obtained after four ultraviolet irradiations and originates from culture 2/4-10 described above. The colony scarcely differs from that of the preceding variant, except that in the center of the bottom surface there are more radial folds (up to 1.7 cm in length) (Figures 5 and 6). This mutant also differs from the preceding one in greater polymorphism of conidia whose ciameter varies from 2.7 to 6.0μ. Conidia 4.5μ in diameter are found most often. However, measurements of thickness of mycelial hyphae, conidiophores and heads are almost the same as in culture 2/4-10.

Mutant T-2.

This mutant is of the same origin as culture T-1 and resembles it in colony shape. Typical of culture T-2 also is the formation of greatly modified heads, resembling the "brush" used to wash test tubes. In these heads the sterigmata are arranged not only

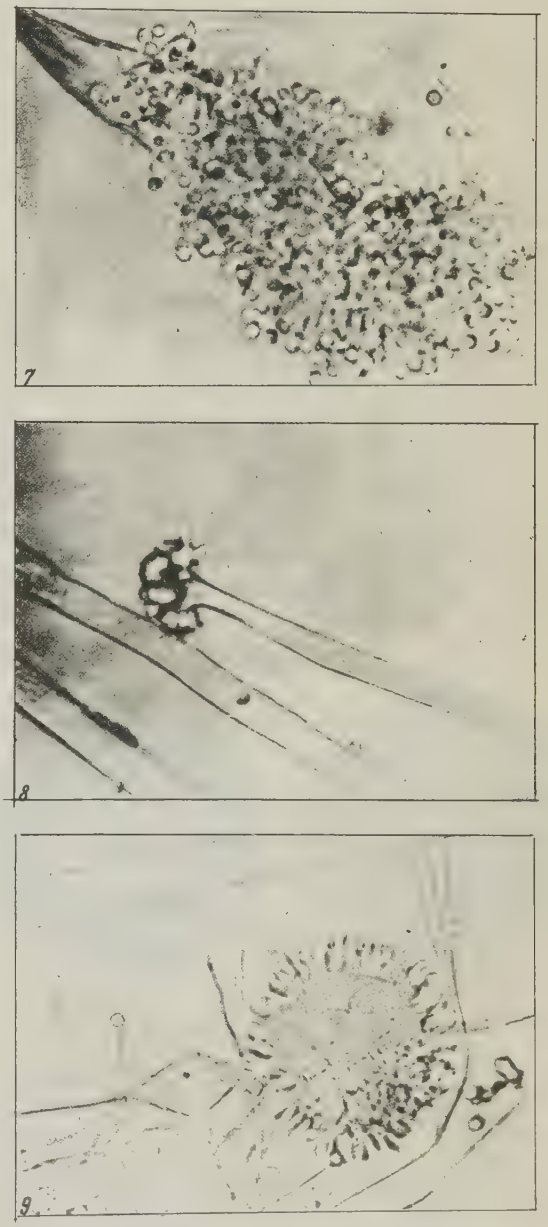


Fig. 7. Deformed conidiophore of variant T-2 in the form of a "brush". 5-day culture on wort agar. Mag. 900X.
Fig. 8. Multiple-head conidiophore in variant T-2. 5-day culture on wort agar. Mag. 90X.
Fig. 9. Conidiophore with normal head. 5-day culture on wort agar. Mag. 90X.

around the head, but also under it, on the upper segment of the conidiophore itself (Figure 7). Sometimes conidiophores with several heads entirely devoid of sterigmata occur (Figure 8). Such deformation of heads is, however, found rather rarely and other heads have the customary appearance (Figure 9). Frequent formation of relatively small conidia 3.8 μ in diameter is also typical of variant T-2.

All three cultures (2/4-10, T-1, and T-2) resemble each other very closely in morphological characteristics: size of colonies and structure of mycelium, conidiophores and heads (Table 1).

Mutant "X".

This culture as well as the two preceding ones was obtained from culture 2/4-10 after four ultraviolet irradiations. This culture differs sharply, however, from the three cultures described above and from the original strain 6/5, chiefly in the appearance of the giant colony. The latter is considerably smaller in this culture, its diameter reaching only 2.5 cm on the fifth day of growth on wort agar. The colony is not always round, sometimes it is slightly oval (Figure 11). The colony surface is covered with tall aerial mycelium. Conidial heads cover the whole colony surface in large numbers, but not uniformly. A dark concentric ring consisting of more densely arranged conidial heads is seen. The colony border is lighter than the center. Conidial heads are brownish black. The entire bottom surface of the colony is greatly wrinkled (Figure 11). The colony border is sharply delimited and slightly uneven (Figure 10 and 11). Variant "X" differs from the original culture and the mutants described not only in the shape and size of the colony. Its cells which form the mycelium are shorter (18-70μ); thickness of mycelial hyphae is more or less constant and equal to 4.1-5.1μ. Mycelium has a large number of branches. Conidiophore thickness is 14.3μ on the average and in this respect the culture differs little from the other variants. Diameter of conidia varies in the range 3.0-5.6μ, i.e. is the same as in culture T-2. However, small conidia are found most often and this distinguishes this variant from the preceding ones.

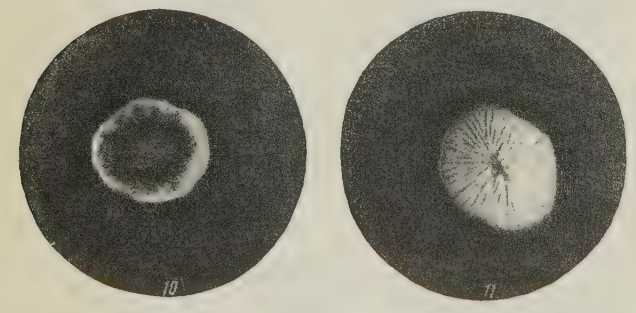


Fig. 10. Mutant "X".
5-day culture, natural size, view from top.
Fig. 11. Same as Fig. 10, view from bottom.

PHYSIOLOGICAL CHARACTERISTICS

Of definite interest is comparative study of the citric acid-forming capacity in the mutants described above. Cultures were grown in 50-ml Erlenmeyer flasks containing 40 ml medium whose composition is indicated above. They were kept in the thermostat for 38-42 hours and after replacement of the medium, for four days. The reaction of the medium on which the fungus pellicle was grown was 4.2 after its acidification with hydrochloric acid. Experimental results are shown in Table 2.

1) In mutants T-1, T-2, and "X" less mycelium, by weight, is formed than in the original culture, but sugar consumption is higher per unit of dry mycelium weight; 2) two of these mutants, namely T-1 and T-2, form more citric acid than the original factory strain, mutant T-1 giving 36% more acid when the citric acid formed is calculated per unit mycelium dry weight; 3) variant 2/4-10 forms approximately the same amount of mycelium and citric acid as the original culture. It should be noted that variant 2/4-10 was also more active earlier, but it was not maintained under suitable conditions and its activity decreased.

Thus we see that the morphologically altered mutants possess at the same time an altered oxidation capacity. The results of more detailed study of their biochemical activity will be given in a subsequent report.

SUMMARY

1. Conidia of the industrial strain of Aspergillus niger 6/5 were irradiated with ultraviolet rays of 2537 A wave length. The dose was $96 \cdot 10^{-3}$ erg per conidium.

2. The method of "step selection" was used, which consists of selecting the mutants arising after the first irradiation with increased capacity to form citric acid. Conidia of these strains were then irradiated and selection of mutants again carried out. These procedures were subsequently repeated.

3. The "step selection" method yields stable variants. Further studies are needed to decide conclusively whether analogous results can be obtained from a single irradiation.

Table 2. Citric Acid Formation in the Mutants Obtained by Ultraviolet Irradiation
(Figures are Averages of Three Determinations)

Culture	Mycelium dry wt. per 40 ml medium	Sugar consumed in %	Citric acid in %	Sugar consumed per g dry mycelium		Citric acid per g dry mycelium in	
				g	%	g	%
Original 6/5	0.7191	17.39	14.5	7.3	100	6.1	100
Mutant 2/4-10	0.7741	16.96	14.7	6.5	89	5.7	94
Mutant "X"	0.6462	17.32	13.9	8.1	111	6.5	107
Mutant T-1	0.5493	17.49	15.1	9.6	132	8.2	136
Mutant T-2	0.6173	17.20	15.3	8.4	115	7.5	123

4. Three stable, morphologically altered strains, T-1, T-2, and "X", were obtained as a result of four irradiations. Variant "X" forms greatly wrinkled and relatively small colonies and is quite different morphologically from the original A. niger 6/5 culture. Mutants T-1 and T-2 were altered relatively little and are similar to one another. Colonies are somewhat smaller, more wrinkled, and form fewer conidia than those of the original strain.

5. Variants T-1 and T-2 produce more citric acid, both in the medium and per unit mycelium dry weight, than the industrial A. niger 6/5 strain. This biochemical feature proved to be stable and was not lost on culture storage. Mutant "X", with a more altered type of colony, produced almost the same amount of citric acid as the original strain.

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OXIDATION OF ORGANIC ACIDS BY ACTINOMYCES VIOLACEUS NO. 719

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The aerobic respiration characteristic of many microorganisms is based chiefly on the utilization of carbohydrates, certain organic acids, and other substances as oxidative substrate.

The oxidation of substrates is known to be carried out by the respiratory system, which contains a heme enzyme and cytochromes in addition to dehydrogenases and intermediate electron carriers. Oxidation processes in plant and animal tissues, as well as in yeasts and bacteria, have been relatively well studied. Studies on the oxidative enzyme systems in actinomycetes are very few.

Godzeski and Stona (1955) studied the enzymes that carry out the tricarboxylic acid cycle in Penicillium chrysogenum Q 176. They found almost all the enzymes necessary to carry out the citric acid cycle, with the exception of the dehydrogenase of α-ketoglutaric acid.

Garner and Koffler (1955) studied the oxidation of the organic acids of the citric acid cycle by Streptomyces griseus. They found that these organic acids are oxidized poorly and the existence of a Krebs cycle was not found in this organism. These organic acids were also oxidized poorly in experiments of Cochrans and Peck (1955). They, however, explain this fact by insufficient membrane permeability. In experiments with ground mycelium the oxidation considerably increased and the authors concluded that Streptomyces coelicolor nevertheless carries out the reactions of the tricarboxylic acid cycle, but it is not known to what extent these reactions participate in the normal oxidative metabolism of actinomycetes.

The available data are contradictory and too inadequate to assert the existence of the tricarboxylic acid cycle in these microorganisms.

On the basis of what has been said it seemed advisable to study the oxidation pathways of organic acids in one of the actinomycetes, Actinomyces violaceus.

METHODS

Actinomyces violaceus was cultured on synthetic medium containing (%): glucose 2; K_2HPO_4 0.05; $MgSO_4$ 0.05; NaCl 0.05; KNO_3 0.1; $CaCO_3$ 0.25 and traces of iron.

To study respiration processes in this organism a 3-day culture was employed in most cases. The washed mycelium was placed in buffer solution of pH 7.0 through which sterile air was passed for 2 hours. This partially removed the substrates of endogenous respiration that might influence the oxygen absorption. Mycelium was filtered through a membrane filter, washed several times with water, and suspended in a definite amount of buffer solution of pH 7.0.

Oxidation of Monocarboxylic Acids.

The rate of oxidation of organic acids by A. violaceus No. 719 was studied in the Warburg respirometer. The suspension under study containing 200 mg mycelium in 3 ml of phosphate buffer of pH 7.0 was placed in the vessels. (Studies were made at pH 7 in order to approximate the experimental conditions under which growth and antibiotic formation occur on Czapek medium.)

To vessel sidearms was added 0.5 ml of the acid under study in 1/30 M concentration, which was introduced into vessels after constant temperature was established in the respirometers. When oxygen absorption was determined the central well contained 0.2 ml 20% KOH and when carbon dioxide liberation was determined it contained water. To control vessels was added a mycelium suspension of the culture without substrate. Experiments were conducted at 25 deg.

The oxidation rate of acetic, lactic, and pyruvic acids is shown in Table 1.

Comparison of the gas metabolism in A. violaceus shows that oxygen absorption is more active in the presence of monocarboxylic acids than in the control without addition of substrate.

A. violaceus absorbs more oxygen in the presence of lactic acid (405 μ l) than with acetic (348 μ l) or pyruvic (236 μ l) acid. It is of interest to note that the microorganism does not completely oxidize these acids to CO_2 and H_2O. The respiratory coefficient is

Table 1. Effect of Acetic, Lactic, and Pyruvic Acids on Respiration of A. violaceus (0.2 g Mycelium in 3 ml Phosphate Buffer pH 7.0. Concentration of Salts of Acids 1/30 M, Duration of Experiment 3 Hours)

Mixture composition	O_2 absorption, µl	CO_2 liberation, µ l	CO_2/O_2	CO_2/O_2 on basis of complete oxidation
Act. violaceus (control)	213.6	109.7	0.50	—
Act. violaceus + sodium acetate	348.0	252.2	0.72	1.0
Act. violaceus + sodium lactate	405.9	217.6	0.53	1.0
Act. violaceus + sodium pyruvate	236.1	165.4	0.70	1.2

this culture is always less than unity in the presence of lactic, acetic, and pyruvic acids. The culture actively oxidizes lactic acid to pyruvic. We isolated pyruvic acid as the 2,4-dinitrophenylhydrazone with melting point 218 deg from the medium containing lactic acid, on which the fermentation took place.

It is also seen from Table 1 that this culture in the presence of pyruvic acid gives off much less CO_2 (165 μ l) compared to the oxygen absorbed (236.1 μ l). The respiratory coefficient here is 0.7. The theoretical ratio $CO_2/O_2 = 1.2$ with complete combustion of pyruvic acid.

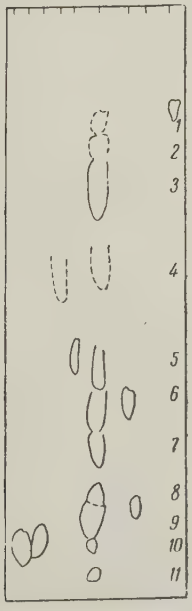
The indicated facts suggest that the decarboxylating enzymes in this microorganism are weakly active. The low respiratory coefficient in A. violaceus shows that pyruvic acid is not completely oxidized and a number of compounds are presumably formed that serve as material for further synthesis of new compounds.

Actually, in paper chromatography of the products of pyruvic acid metabolism we succeeded in detecting a number of organic acids (figure). Our further studies showed that A. violaceus forms these acids also from glucose.

It is seen from the figure that from pyruvic acid alone A. violaceus forms ten organic acids. Of these we succeeded in identifying the following acids: malic, glycolic, lactic, succinic, fumaric. The others we did not succeed in identifying. Citric acid was not found during fermentation of the medium in the presence of pyruvic acid. Nor does this culture form citric acid from oxaloacetic acid in the presence of pyruvic acid.

Pyruvic acid is an intermediate product in breakdown of carbohydrates, proteins (via alanine), and fats. More than seven pathways of pyruvic acid conversion are now known in microorganisms, plants, and animals.

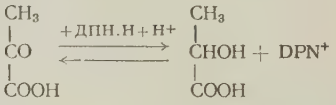
According to current data, pyruvic acid oxidation in the di- and tricarboxylic acid cycles occurs through



Chromatogram sample:
1–4) Unknown acids; 5) malic; 6) glycolic; 7) unknown; 8) lactic; 9) pyruvic; 10) succinic; 11) fumaric acids.

acetyl CoA. There has recently been found an enzyme system, pyruvic oxidase, which catalyzes the conversion of pyruvic acid through acetyl CoA (Ochoa, 1951). This enzyme system contains coenzyme A, cocarboxylase, diphosphopyridine nucleotide (DPN), Mn^{++}, and lipoic acid. Pyruvic acid in the A. violaceus culture is probably oxidized by pyruvic oxidase to acetyl CoA and through the latter succinic acid is synthesized.

It should be noted that A. violaceus is also able to reduce pyruvic acid to lactic; consequently this microorganism contains lactic dehydrogenase, which in the presence of DPN^+ reduces pyruvic acid to lactic according to the scheme



This is confirmed in the experiment where A. violaceus was grown with pyruvic acid. Formation of lactic acid from pyruvic is shown on the chromatogram. A. violaceus also forms pyruvic acid from lactic. This process is reversible.

Oxidation of Di- and Tricarboxylic Acids.

Studies on oxidation of succinic, malic, and citric acids were carried out in the Warburg respirometer. The experimental setup was the same as in the preceding experiments. Oxidation rate of these acids is shown in Table 2.

From Table 2 it is seen that succinic acid is oxidized most actively of the acids studied. A. violaceus in the presence of succinic acid absorbed 391 μ l oxygen and gave off 214 μ l carbon dioxide. This culture also oxidizes malic acid, but the oxidation proceeds much more weakly than that of succinic acid; it oxidizes citric acid very weakly.

It is evident from Tables 1 and 2 that A. violaceus in the presence of all the acids studied absorbs considerable amounts of oxygen (particularly in the presence of succinic acid) and gives off considerably less carbon dioxide than the theoretically possible amount.

This fact indicates that the decarboxylating enzymes in A. violaceus are weakly active. Since this microorganism does not oxidize the organic acids studied to CO_2 and H_2O, they are probably first oxidized and then the oxidized compounds decarboxylated to certain reduced compounds from which amino acids, protein compounds, and antibiotics can be synthesized. A. violaceus grows well on Czapek medium containing succinic acid and forms antibiotic in an amount up to 1,000,000 conventional units.

Studies were made on the activity of lactic and succinic acid dehydrogenases in A. violaceus. These dehydrogenases were determined by their ability to reduce methylene blue in special vacuum tubes. Methylene blue was decolorized in 30–40 minutes. Methylene blue without these substrates is not decolorized during this time.

Cytochrome Oxidase.

It is known in the literature that succinic dehydrogenase participates in an oxidative system in which

Table 2. Respiration of A. violaceus in the Presence of Succinic, Malic, and Citric Acids (0.2 g Mycelium in 3 ml Phosphate Buffer pH 7.0; Concentration of Salts of Acids 1/30 M, Duration of Experiment 3 Hours)

Mixture composition	O_2 absorption, μl	CO_2 liberation, μl	CO_2/O_2	CO_2/O_2 on basis of complete oxidation
Act. violaceus (control)	145.7	91.1	0.62	—
Act. violaceus + sodium succinate	391.2	214.5	0.55	1.14
Act. violaceus + sodium malate	279.8	117.6	0.42	1.33
Act. violaceus + sodium citrate	162.5	104.0	0.64	1.5

Table 3. Effect of Cyctochrome C and Succinic Acid on Oxygen Absorption in A. violaceus (Duration of Experiment 4 Hours)

Mixture composition	O_2 absorption, μl
Act. violaceus (control)	184.2
Act. violaceus + sodium succinate	403.1
Act. violaceus + cytochrome c	252.6
Act. violaceus + sodium succinate + cytochrome c	677.0
Act. violaceus + sodium succinate + cytochrome c + KCN M/100	70.4

the terminal enzyme is cytochrome oxidase.

To establish the presence of cytochrome oxidase, we used specific substrates and inhibitors. Cytochrome oxidase activity was determined from oxygen absorption in the Warburg apparatus. Cytochrome c was the substrate. Hydroquinone is known to be employed as reducer of cytochrome c in most cases to determine cytochrome oxidase in plant tissues (Mikhlin and Kolesnikov, 1947). On adding hydroquinone to the cytochrome, strong inhibition of respiration was noted in A. violaceus. Hydroquinone was hence unsuitable for determining cytochrome oxidase in this microorganism.

Then it was decided to employ succinic acid to reduce cytochrome c in determining cytochrome oxidase, on the basis that A. violaceus vigorously dehydrogenates succinic acid. As was to be anticipated, succinic acid is an excellent substrate for reducing cytochrome c. It is seen from Table 3 that sharp increase in amount of oxygen absorbed is observed on adding succinic acid and cytochrome c to the culture.

Consequently, cytochrome oxidase can be judged to be present in A. violaceus from the increase in oxygen absorption in the respirometers after addition of cytochrome c and succinic acid to the suspension of mycelium. The amount of oxygen absorbed increases to 677 μ l. It should be noted that oxygen absorption increases compared to control even without addition of succinic acid. Possibly A. violaceus forms certain substances that reduce cytochrome c. This was observed also in experiments of Tauson (1958) in which cytochrome c was weakly oxidized without addition of reducing agent.

It is of interest to note that potassium cyanide in 1/100 M concentration inhibits cytochrome c oxidation, but here there always remains a residual respiration. This residual respiration must be attributed to the flavoprotein enzymes. The A. violaceus culture we studied contains cytochrome oxidase. It may be suggested that succinic dehydrogenase participates in hydrogen transfer to the cytochrome oxidase system according to the scheme:

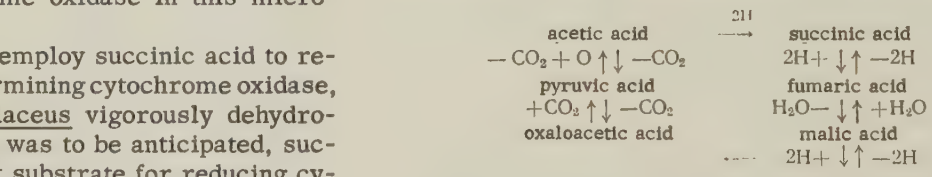
succinic acid → succinic dehydrogenase → cytochrome oxidase → O_2

DISCUSSION OF RESULTS

It is known that pyruvic acid is an intermediary product in the conversion of carbohydrates, fats, and proteins and that the di- and tricarboxylic acid cycles are of great importance in the oxidative metabolism of microorganisms.

The investigations on oxidation and conversion of pyruvic acid in A. violaceus have shown that this microorganism forms the dicarboxylic acids succinic, malic, and fumaric from pyruvic acid. The tricarboxylic acids aconitic, isocitric, and citric cannot be synthesized by A. violaceus either from pyruvic or from oxaloacetic acid in the presence of pyruvic.

On the basis of the facts obtained, it must be supposed that pyruvic acid oxidation proceeds through the dicarboxylic acid cycle according to the scheme:



The following facts are in favor of this scheme: when A. violaceus is grown on medium containing pyruvic acid, the individual links in the cycle of dicarboxylic acids—malic, succinic, fumaric—are found. The dehydrogenases of most of these acids are also found. Such a pathway of acetic acid conversion was demonstrated for the first time in mold fungi by Butkevich and Fedorov (1930). A little later Wieland and Sonderhoff (1932) also observed formation of succinic acid from acetic in bread yeasts.

Our studies have shown that A. violaceus actively oxidizes cytochrome c in the presence of succinic acid. Probably cytochrome oxidase is the terminal oxidative enzyme system. Respiratory coefficients of A. violaceus in solutions of the organic acids studied are 30–50% below the theoretical. The coefficients indicate that the organic acids are not oxidized completely to carbon dioxide and water. Intermediate products are probably formed from which amino acids, proteins, lipoids, antibiotics, and other intracellular substances are apparently synthesized.

SUMMARY

1. Actinomyces violaceus oxidizes succinic and lactic acids actively. Pyruvic, acetic, and malic acids are oxidized less actively and citric acid very slightly.

2. This microorganism forms ten acids from pyruvic: succinic, fumaric, malic, glycolic, lactic, and four as yet unidentified acids. Citric acid cannot be synthesized from pyruvic, or from oxaloacetic acid in the presence of pyruvic. These facts indicate that this microorganism oxidizes pyruvic acid via the dicarboxylic acid cycle.

3. A. violaceus contains most of the enzymes that oxidize dicarboxylic acids, as well as enzyme systems that convert pyruvic acid to lactic.

4. A. violaceus actively oxidizes cytochrome c in the presence of succinic acid. Cytochrome oxidase is probably the terminal link in the oxidative system of this microorganism.

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RESPIRATION OF GREATLY MODIFIED ASPERGILLUS NIDULANS VARIANTS OBTAINED BY ULTRAVIOLET IRRADIATION

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Greatly modified Aspergillus nidulans variants, obtained by ultraviolet irradiation, with marked degenerative changes in mycelium structure had lost proteolytic activity (Verbina, 1958) and required a higher degree of aeration and displayed more requirements for constituents in the nutrient medium than the original culture (Verbina, 1959). In view of these serious disturbances in metabolism, it was of interest to determine to what extent the respiration activity had been modified in the degenerate variants.

It is known in the literature that in certain wrinkled forms of fungi and yeasts a shift in oxidative capacity occurs (Imshenetskii, 1950; Karasevich, 1958; Kasatkina, 1952). Here the oxidation of glucose proceeds very vigorously but does not go to completion and remains in the beginning stages, with resultant accumulation in the medium of intermediate products in considerable quantity which are not oxidized further.

It might be assumed that in the irradiated A. nidulans variants some enzyme systems were damaged considerably, but that others were activated in compensation. The existence of "correlation" of this type is pointed out by Foster (1949) in giving one of the possible explanations of the stimulation of certain physiological processes in mutants.

On the basis of this assumption, a study was made of the respiration activity in the greatly modified variants of A. nidulans. On the other hand, in view of the observations (Verbina, 1959) that under certain cultural conditions which maximally satisfy the increased requirements of the irradiated organism, the deficiency in the mutant is eliminated and the growth activity of original culture and mutant becomes equal, it was of interest to determine what the relation is between the respiration and the changes taking place in the physiology of the mutants in this case.

METHODS

The work was done with the greatly modified variant n/12, obtained by ultraviolet irradiation of the parent A. nidulans culture. Respiration activity was determined manometrically in the Warburg apparatus. In the first series of experiments the respiration of these strains was studied in surface culture. The fungi were grown on beer wort, 7 deg Balling, in 250-ml Erlenmeyer flasks. Amount of medium was 70 ml. Seedings were made with filtered conidia in the amount 400-500 thousand per ml medium. On the third day the fungus pellicles were removed, washed several times with water, and pressed between sheets of filter paper.

In the beginning of the study, equal amounts of both cultures were ground for 2 minutes until a homogeneous mass was obtained, which was added with a pipette to respiration vessels. The determination of respiration activity showed, however, that O_2 absorption was negligibly small in this case. Evidently grinding of the mycelium resulted in inactivating respiratory enzymes. In further work, therefore, 40 mg of wet pellicle of each culture, without grinding, was added to each vessel. The same amounts of fungus pellicle were simultaneously placed in vials for determination of dry weight. Vials were dried to constant weight at 100-105 deg. The dry weight of these batches was about 10 kg. The mycelium was placed in the vessel to which was added 1 ml phosphate buffer (pH 5.59). To the vessel sidearm was added 0.5 ml of respiration substrate. To control vessels in which endogenous respiration of mycelium was determined, 0.5 ml water was added instead of substrate for oxidation. To the alkali well was added 0.25 ml 20% KOH.

In the second series of experiments, fungi were cultured under conditions where equal biomass production in original culture and mutant was observed, i.e. in submerged culture on a shaker with high rotation rate (250 r.p.m.). Fungi were grown in 250-ml flasks containing 70 ml beer wort, 7 deg Balling; seed material in the amount 2 million conidia per ml medium was added to nutrient media in these experiments. Duration of cultivation was 20 hours. By this time a thick suspension of submerged mycelium had formed in the medium, which was readily taken up in a pipette. This made it possible to add the same amount of mycelium to each respiration vessel. In order to eliminate the inaccuracy in respiration experiments associated with the high degree of endogenous respiration in the fungi, that distorts results on glucose oxidation, the mycelium that grew out was carefully washed free of medium. Mycelium was centrifuged twice in phosphate buffer and placed on a shaker in flasks containing a fresh portion of buffer for 5-6 hours. Aeration of the mycelium washed free of nutrient medium is recommended to remove readily oxidizable reserve substance in the fungus and to reduce the endogenous respiration (Stevenson, 1951; Umbreit et al., 1951). Then the mycelium is centrifuged from the liquid and a thick suspension made from it in phosphate buffer, such that 1 ml corresponds to approximately 10 mg biomass dry

weight. To the main body of each vessel 1 ml of such a suspension was added. Other experimental conditions in the Warburg apparatus were the same as in the first series of experiments in surface culture. Two or three parallel manometers were employed for each experiment variant. Readings were taken every 30 minutes. Q_{O_2} (μl 02/hour/mg dry weight) was calculated from the data obtained.

EXPERIMENTAL

1. Respiration of original A. nidulans culture and variant n/12 in surface culture.

The studies were begun by determining endogenous respiration, i.e. respiration of the washed mycelium in the absence of oxidizable substrate. The experiments showed that both cultures had considerable endogenous respiration, but in variant n/12 it was appreciably less active than in the original culture (Figure 1). In this connection it was necessary to check to what extent the oxidative capacity of variant n/12 had changed. Glucose (0.1 M) was used as oxidizable substrate. These experiments showed that variant n/12 oxidizes glucose less actively than the original culture (Figure 2). Since the amount of oxygen consumed by the mutant was less than in the original culture, this might indicate that complete oxidation of glucose to CO_2 and water in the respiratory process of the mutant does not occur in this case. It was suggested that this is due to the fact that as a result of ultraviolet irradiation the enzymes effecting the beginning stages of respiration become more active than in the original culture. In that case the variant n/12 should, in oxidizing glucose, accumulate products of the incomplete oxidation of glucose in the medium, for instance organic acids.

For this purpose experiments on glucose oxidation were set up in the Warburg apparatus, in which the ratio was determined between the amount of oxygen absorbed during the experiment and the amount of glucose disappearing from the solution in this time. Glucose was determined by the micromethod of Berry (Belozerskii et al., 1951). If variant n/12, with reduced respiration, consumed as much sugar as the original culture with active respiration, this would serve to confirm the suggestion regarding accumulation of intermediate products in the form of organic acids.

Table. Consumption of Oxygen and Glucose in Respiration of Original A. nidulans Culture and Variant n/12

Expt. no.	Culture	O_2 consumed, l/hr/mg dry wt.				Glucose consumption	
		endogenous	%	on glucose	%	mg/mg dry wt.	%
1	Original	41.5		88.7		7.8	
2		37.6		75.1		7.3	
3		45.9		90.1		8.1	
Average		41.7	100	84.6	100	7.7	100
1	n/12	30.9		62.0		5.5	
2		26.0		53.8		4.8	
3		33.5		68.3		6.0	
Average		30.1	70.0	60.0	70.0	5.2	67.0

The results of these experiments are shown in the table. The numerical data from these experiment confirm those from the preceding experiments; the amount of oxygen consumed by variant n/12 in glucose oxidation is 70% that in the original culture. Consumption of sugar by variant n/12 calculated per unit dry weight was also found to be lower (by 33%) than in the original culture. Moreover, we could not detect appreciable acidification of the fluid in respiration vessels at the end of the experiment, which would indicate accumulation of organic acids. Consequently our suggestion of an activation in this case of certain respiratory enzymes in the mutants, resulting from ultraviolet irradiation, was not confirmed. These results are in conformity with previously obtained data, that variant n/12 is an organism with reduced biological potential. In surface culture, it manifests deficiency not only in growth, but also in respiration and oxidative capacity. Since previous observations showed that the deficiency of this

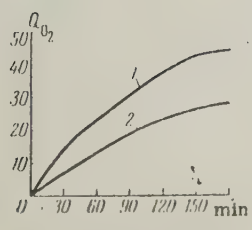

Fig. 1. Endogenous respiration of original B. nidulans culture and variant n/12.

1) Original culture; 2) variant n/12.

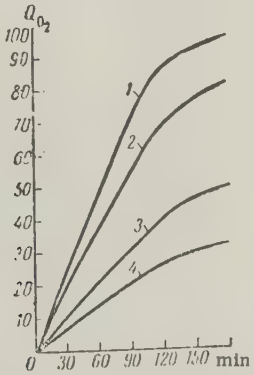

Fig. 2. Respiration activity of A. nidulans and variant n/12 in surface culture.

1) Glucose oxidation by original A. nidulans culture; 2) glucose oxidation by variant n/12; 3) endogenous respiration of original culture; 4) endogenous respiration of variant n/12.

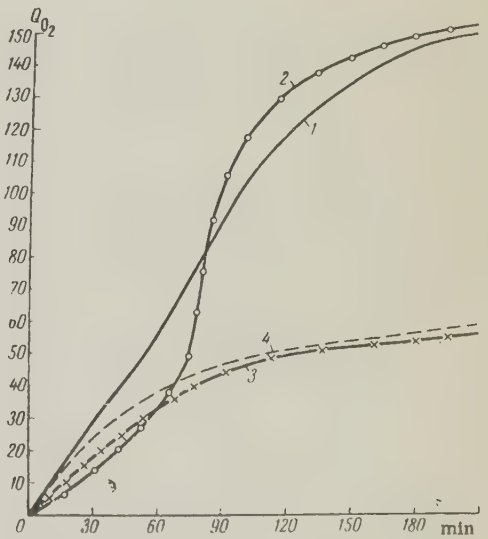

Fig. 3. Oxidative capacity in original A. nidulans culture and variant n/12 in submerged culture.

1) Glucose oxidation in original culture; 2) glucose oxidation in variant n/12; 3) endogenous respiration in original culture; 4) endogenous respiration in variant n/12.

organism with respect to growth could be overcome in submerged culture on a rapid shaker and complete medium, it was necessary to determine what happens to respiration under these conditions.

2. Respiration of original A. nidulans culture and variant n/12 in submerged culture.

Manometric data on endogenous respiration and glucose oxidation in submerged cultures of A. nidulans and variant n/12 are shown in Figure 3. The curves obtained show that the endogenous respiration of original culture and variant n/12 is equally active throughout the entire experiment (3.5 hours). Glucose oxidation is the mutant at first lagged somewhat behind the rate of the original culture; by the end of the experiment the mutant's oxidative activity reached the same level as in the original culture, and in some experiments even exceeded it. Sugar consumption was the same in both cultures (Figure 4).

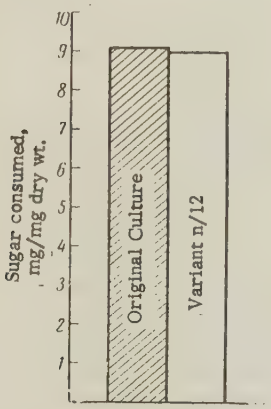

Fig. 4. Glucose consumption in respiration of original A. nidulans culture and variant n/12.

1) original culture; 2) variant n/12.

Comparing previously obtained data on the physiology of greatly modified A. nidulans variants with the results of this study, the following conclusions can be made.

The deficiency in the greatly modified variant n/12, expressed in low production of biomass compared with the original culture when grown on solid nutrient medium or as a pellicle on the surface of liquid medium, can be overcome by setting up more favorable cultural conditions. When the fungi are grown on complete natural medium on a shaker with rapid rotation rate, growth of the mutant and of the original culture is equally active.

Under conditions where poor biosynthesis of mycelium in mutant n/12 was noted, there was also observed reduced endogenous respiration and low oxidative activity compared to the original culture. But when biomass production was the same in both cultures in submerged culture, the increased growth activity of the mutant was accompanied by appreciable increase in respiration activity and in activity of oxidative enzymes. This once again confirms the existence of an extremely close relation between growth and respiration. The more actively this organism grows, under given stable conditions, the more actively it respires, and vice versa. This situation apparently extends also to experimentally obtained organisms having reduced biological potential. In the present case the disturbances in the organism produced by ultraviolet irradiation proved to be reversible, in spite of the fact that variant n/12 under ordinary laboratory maintenance conditions has retained the properties it acquired after treatment with ultraviolet light for five years. Study of the requirements of the mutants obtained can provide much of value for the successful restoration in them of one sort of damage or another.

SUMMARY

1. In the greatly modified variant n/12 from Aspergillus nidulans, the endogenous respiration in surface culture is 30% less than in the original culture.

2. Oxygen consumption in glucose oxidation by variant n/12 is less than in the original culture. Sugar consumption per unit dry weight is 33% lower in variant n/12 than in the original culture.

3. The reduced respiration in surface culture indicates a reduced biological potential in variant n/12.

4. Endogenous respiration and oxidative capacity in variant n/12 and in the original culture are the same in submerged culture on a rapid shaker and complete nutrient medium. Sugar consumption in both cultures is the same.

5. The deficiency in respiration rate in variant n/12 can be eliminated by proper cultural conditions.

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SOME PHYSIOLOGICAL FEATURES OF ASPOROGENOUS MUTANTS OF BACILLI

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Various asporogenous strains of bacilli have been randomly isolated and described by different authors (Chamberland and Roux, 1883; Knaysi, 1935; Lehmann (cited by Pringsheim), 1910; etc.). But, except for one study comparing respiration and autolysis in an asporogenous strain of Bacillus megaterium with a sporogenous strain (Tinelli, 1955), the morphological and physiological properties of asporogenous bacilli have not been compared with the original sporogenous strains. The present paper deals with the isolation of asporogenous mutants of bacilli and a comparison of some of their properties with the original forms.

MATERIALS AND METHODS

The starting material was B. subtilis S2 and its biochemical mutants S114 (histidine), S503 (leucine), and S492 (thiamine), and B. megaterium 46.

The bacilli were grown on synthetic medium (medium 1) of the following composition: $(NH_4)_2SO_4$ 5 g, KH_2PO_4 3 g, NaCl 5 g, glucose 5 g, asparagine 1 g, distilled water 1000 ml, 10% solution of $MgSO_4 \cdot 7H_2O$ 2 ml, 1% solution of $MnSO_4$ 0.2 ml, 2% solution of $FeCl_3 + CaCl_2$ 1 ml, pH 7.2; and on peptone medium (medium 2): peptone 10 g, glucose 10 g, yeast extract 1 g, NaCl 5 g, distilled water 1000 ml, pH 7.2; media were employed either as liquids or with 1.5% agar. Total nitrogen was determined colorimetrically in 2.5 ml of culture, dipicolinic acid (DPA) chromatographically. The DPA which served as chromatographic standard was isolated and identified by the method of Perry and Foster (1955) from spores of strain S2. Chromatography was conducted on paper in a mixture of ether:water:formic acid 95% (84:4:6) at 14 deg. Ascending chromatograms were dried 10 minutes in an air current. DPA on chromatograms was identified by means of a freshly prepared solution of 0.5% $FeSO_4 \cdot 7H_2O$ and 10 ml concentrated acetic acid in 100 ml of water. This reagent was employed by Jansen et al. (1958) to determine DPA colorimetrically. DPA gives with this solution on chromatograms a brick-red spot, R_f 0.84; the color is relatively unstable and disappears in 20–30 minutes. Organic acids on the chromatograms were developed with glucose and $AgNO_3$ (Hais and Macek, 1954).

Obtaining Asporogenous Mutants.

Asporogenous mutants were obtained by irradiating cultures with ultraviolet rays.

A washed suspension of B. subtilis spores in physiological solution was heated for 8 minutes in a boiling water bath to kill the non-spore forms and cooled rapidly. The suspension was irradiated for 2–5 minutes with the ultraviolet lamp Original Kanau at a distance of 20 cm from the radiation source in Petri dishes with constant stirring by a magnetic stirrer. There remained 0.1–0.01% of living cells. The irradiated suspension in 0.2 ml amount was seeded on agar medium 1 and cultivated at 35 deg. After two days spore formation began and colonies were brown. The brown color of spore-forming colonies depends on the Mn content in the medium and becomes more intense with increased content. Among the brown colonies could be detected white colonies that on prolonged cultivation retained their white color, typical of young colonies containing only vegetative cells. It was found that most of the white colonies are those of asporogenous mutants.

Among the white colonies a small percentage were sporogenous forms in which the typical spore color was absent. An average of 0.3% stable asporogenous mutants was obtained in the various experiments. The selection of asporogenous mutants was greatly simplified by use of this technique. To obtain mutants from B. megaterium, strain 46 was employed, whose colonies become red-brown during spore formation and is particularly evident on glucose-peptone agar; on synthetic agar the colonies only become a dirty pink and colony color is not affected by the Mn content. Preparation and irradiation of suspensions were the same as in experiments with B. subtilis, except that suspensions were heated prior to irradiation for 8 minutes at 80 deg. Colonies of the asporogenous mutants were at first white and then yellowish, readily detectable among the red-brown colonies of the original strain.

Morphological and Cultural Features of Mutants.

The colonies of the asporogenous mutants of B. subtilis on glucose-peptone agar have a characteristic appearance. Three different types of growth were observed, which could change from one to another.

1. R-form, colonies flat with granular surface, almost the same as young vegetative colonies of the sporogenous forms.
2. R-form, colonies transparent, wrinkled.
3. S-form, colonies smooth, lustrous, somewhat sprawling (Figures 1–4).

The mutants obtained by cultivation with dichromate formed only the S-form and in the course of a year of cultivation did not change into other forms; in the other

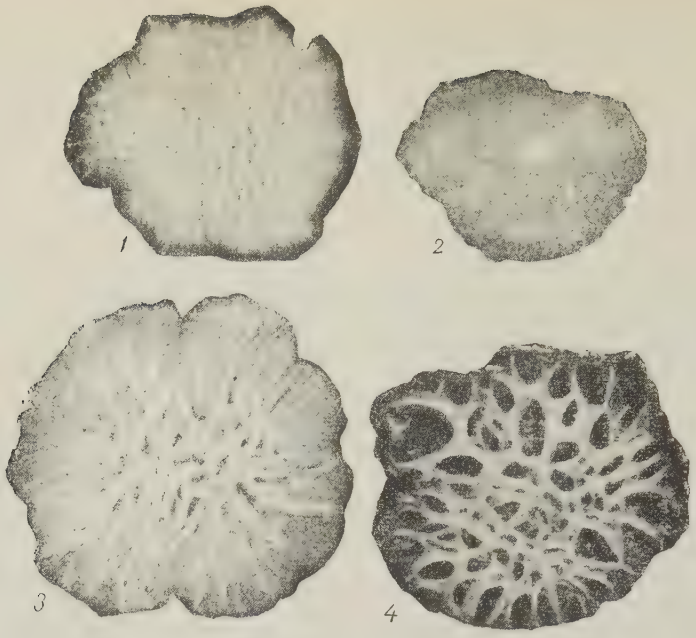

Fig. Colonies of B. subtilis (culture age 18 hours)
1) Spore-forming strain; 2) asporogenous mutant, flat type of growth;
3 and 4) asporogenous mutant, wrinkled type of growth.

mutants, on the contrary, changes of one form into another were often observed. Some B. megaterium mutants did not differ from the original strain 46 either in colony form or cell form; however, quite often a mutant was found (strains 46/57, 46/109, 46/115) that represented a special type of mutation. Cells of this mutant were smaller than in the original strain, and towards the end of active growth seemed to begin to turn into spores (pro-spore stage). Growth stopped at this stage and cells started to be lysed. A substance was apparently formed during the autolysis, which strongly inhibited growth, since on transferring such cultures to fresh agar plates the bacilli either did not grow or grew very poorly in the first transfers.

The bacilli began to grow only in places more remote from the site of application of the seed material, where only insignificant amounts of it were present. Thus, the growth of this mutant on plates consisted of two zones: a sterile zone, where only cells of anomalous form grew poorly, and a zone of luxuriant growth of bacilli with morphology normal for the mutant. Cultures of this mutant proved to be unstable and soon died. Spore formation in the mutant 46/104 of B. megaterium was strongly inhibited by some component of peptone; spore formation proceeded normally in the absence of peptone or on synthetic medium.

Two different types of partial inhibition of spore formation were observed among B. subtilis mutants. Maximal temperature of spore formation in strain 492/323 was 31–32 deg, while in the original strain it was 53 deg. At room temperature active spore formation was observed in this strain, but above 32 deg the culture grew just like an asporogenous one. The maximal temperature of growth, 56 deg, remained the same as in the original strain.

Colonies of B. subtilis strains 114/1, 114/344, and 503/510 had the appearance of asporogenous mutants,

Table. Reduced Viability in Asporogenous Cultures of B. subtilis Mutants

Mutants	Culture age			
	14 hrs		46 hrs	
	no. of viable bacteria, $X10^9$, ml	bacterial N, μ g/ml	no. of viable bacteria, ml	bacterial N, μg/ml
Asporogenous 503/464	5.8	65	2.8×10^6	27
Sporogenous S530	7.0	91	7.9×10^9	67
Asporogenous S2/250	7.4	88	5.3×10^7	50
Sporogenous S2	6.9	92	7.1×10^9	69

but there was always a certain quantity of spores in them after 3–4 days. These spore-forming cells did not arise as a result of reversible mutation. This is indicated by the fact that cultures were isolated from spores separated from vegetative cells by heating for 10 minutes at 100 deg by the method of Lederberg (1954), which had the appearance of asporogenous strains with slow and weak spore formation.

As was noted, some asporogenous strains did not keep well in culture and soon autolyzed. The table gives certain data from experiments on comparative viability in sporogenous cultures and asporogenous mutants. Bacilli were grown on a shaker at 35 deg on medium 2 for 14 and 46 hours.

The table shows that the viability of asporogenous mutants is considerably lower than the sporogenous forms. Our data on B. subtilis mutants fully confirm the observation of Tinelli (1955) on autolysis of asporogenous mutants. Studies on B. megaterium show that this autolysis is accompanied by sharp reduction in number of viable cells.

Formation of Dipicolinic Acid.

The presence of DPA was determined in asporogenous strains in the stationary growth phase by a modified method of Perry and Foster (1955). Strains of B.

subtilis and B. megaterium were grown on agar medium 2 for 30 hours. Spore formation in the control sporogenous strains began under these conditions in 13–15 hours. S-strains and spores were washed from the surface of the medium with distilled water, washed by centrifuging, and again suspended in distilled water. Films of the asporogenous R-strains were washed from the agar surface and divided into two portions, which were suspended and washed in the centrifuge with distilled water. One portion was used to determine DPA, the other to determine dry weight. The volume of the suspension of bacteria or bacterial film in distilled water was made up to 10 ml, and heated 20 minutes in the autoclave at a pressure of 1 atm. Here the DPA was extracted and the bacterial film dissolved. The suspension was cooled and for further analysis an amount was taken equivalent to 0.15 g of dry weight of bacilli or spores. pH of the sample was brought to 1.0 with strong H_2SO_4 and the suspension shaken with ether in the ratio 1:15. After evaporation of the ether the extract was dissolved in 0.2 ml distilled water. A saturated solution of DPA was thus obtained from the spore extracts. To chromatograms 0.2 ml of extracts from mutants was applied and only 15-20 μl of extracts from spores, which was sufficient to obtain distinct spots of DPA. In order to determine the DPA given off in the nutrient medium, bacilli were cultured in a thin layer of media 1 and 2 for 18–48 hours on a shaker. The nutrient medium was removed from the bacterial mass by centrifuging, and an amount of medium containing about 0.15 g of bacterial dry matter was evaporated to small volume in the water bath; pH was brought to 1.0 with H_2SO_4 and the total volume to 5 ml. Subsequently the samples were treated in the same way as those from bacilli. Control experiments showed that this method can separately determine DPA added to the culture fluid.

DPA formation was studied in 19 asporogenous strains of B. subtilis and in 5 strains of B. megaterium; controls were the original sporogenous strains. No DPA was found in the asporogenous strains tested, either in bacilli or in nutrient medium, or more precisely, the content in bacilli was less than 0.03% of dry matter, since sensitivity of the method is about 40 μg DPA. Therefore the DPA content in asporogenous strains is a hundred times less than in sporogenous ones. The temperature mutant 492/323 formed DPA at 28 deg but not at 38 deg.

DISCUSSION OF RESULTS

Spore formation is one of the processes in morphogenesis of bacilli. It is accompanied by increased physiological activity of the bacilli, manifested in increased respiration (Tinelli, 1955), synthesis of proteins and nucleic acids (Malek et al., 1953), changes in enzyme composition (Hardwick and Foster, 1953), synthesis of dipicolinic acid (Powell, 1953), Ca absorption (Vinter, 1950). It is entirely possible that in the morphological mutants from bacilli, unable to form spores, one of the synthetic processes indicated that accompany sporogenesis is inhibited, similarly to the inhibition of synthesis of compounds required for active growth in the usual biochemical mutants. It is interesting to note in regard to this that no DPA was found in any of the asporogenous mutants we studied. Possibly in these mutants some reaction in DPA synthesis is inhibited; this suggestion is the subject of further study.

It seems to us that the data from our experiments also indicate a certain physiological significance of spore formation to the bacillus. Certain authors note that the spore-formation process in many cases is just as sensitive to environmental influences as the vegetative growth, or more so. On this basis Lamanna (1952) doubted whether the process of spore formation is of any biological significance at all to the bacillus. It is evident from the experiments with asporogenous forms of bacilli that spore formation is important in maintaining the viability of bacilli under environmental conditions where there occur retardation and gradual cessation of growth and autolysis, when the medium becomes unsuitable for further growth owing to accumulation in it of cellular products of the bacilli themselves.

SUMMARY

1. Asporogenous mutants from B. subtilis and B. megaterium were obtained by ultraviolet irradiation. A simple method for selecting asporogenous colonies is described, based on the brownish color of sporogenous colonies in the B. subtilis strain on synthetic medium with increased Mn content, and on the red-brown color of sporogenous colonies of B. megaterium 46 on peptone agar containing yeast extract, while asporogenous colonies remained colorless.

2. Cultures with partial inhibition of spore formation were found among the asporogenous mutants isolated. A B. subtilis mutant was isolated whose maximal temperature of spore formation had decreased from 51 to 32 deg, but maximal temperature of vegetative growth remained the same as in the original strain. A B. megaterium mutant was obtained in which spore formation was inhibited by peptone.

3. No dipicolinic acid was found, either in cells or in nutrient medium, in 19 asporogenous B. subtilis strains and 5 B. megaterium strains.

4. Asporogenous strains are relatively unstable, readily undergo lysis, and die. Comparison of sporogenous and asporogenous bacilli showed that the viability of asporogenous forms is soon lowered, while spore formation takes place in the former.

5. It is suggested that spore formation is of significance as a factor in stabilizing the cell against its own autolytic enzymes when growth is partially retarded owing to nutrient deficiency.

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GROWTH CHARACTERISTICS OF A YEAST FUNGUS OF THE FAMILY SCHIZOSACCHAROMYCETACEAE

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Dividing schizosaccharomycete cells were found in the budding yeast mass in fermentation vats of the experimental installation for fermentation without separation at the Leningrad Hydrolysis Plant in 1957.

The dividing yeast cells were isolated from the yeast mass by V. A. Utenkova, M. K. Raitseva, and M. M. Abramovich.

The yeast was maintained for a year in the laboratory on wort agar and was not studied in detail until 1958. In the course of the study asci with four spores were found in 10-30-day cultures on wort agar.

The spores are formed in parthogenic asci, i.e., without fusion of cells, and on release from the ascus do not disperse but have a characteristic arrangement resembling a mulberry (Figure 1).

According to the classification of yeasts by Kudryavtsev (1954), dividing yeasts belonging to the Schizosaccharomycetaceae are represented by two genera, Schizosaccharomyces and Octosporomyces. Typical of both genera is the formation of spores in sexual asci following isogamous fusion of the yeast cells, which was not observed in the yeast we isolated.

According to data in the literature, yeasts sometimes become asexual when grown under laboratory conditions, i.e., they lose the capacity for conjugation, and the spores are then formed in parthogenic asci (Krasil'nikov, 1935, 1943).

Methods to stimulate the sexual process in yeasts were proposed by Krasil'nikov—joint cultivation with bacteria, growth on media containing an aqueous extract of barley sprouts, seeding on wort agar.

We did not succeed in stimulating the fusion of cells in the yeast isolated either by repeated seeding on wort agar or by seedings on agar containing barley-sprout extract. We observed accelerated spore formation in these experiments, but the spores always formed without previous fusion of cells.

The special features in the growth of the yeast fungus we isolated prompted us to study the culture in greater detail.

A yeast culture was isolated from a single cell by the method of Lindner.

The cells of a 24-hour culture of the isolated yeast on wort agar are ellipsoidal with average dimensions of $9 \times 6\ \mu$ to $12 \times 9\ \mu$.

During fermentation in the hydrolysis wort the yeast is rod-shaped with dimensions of $36 \times 6\ \mu$ to $60 \times 9\ \mu$.

In studying the vegetative reproduction on wort agar in a ш-shaped chamber according to Peshkov, cells 80-100 μ in size are observed that contain two to four septa. This elongated yeast cell divides into a number of cells of varying length.

By the 10th-30th day of growth on wort agar at room temperature, the yeast culture forms asci with spores arising without prior fusion of cells. Most of the cells proceed to the arthrospore state.

We followed the process of spore germination, using the ш-shaped chamber. Observations were made of the same spores at 35 deg.

It should be noted that under the conditions in the W-shaped chamber only single spores germinate; in 10-15 hours the spores increase in size, elongate, and are converted to yeast cells. Of four observed spores, only one or two as a rule are converted to vegetative cells, with no previous fusion of the spores (Figure 2).

We studied the nature of arthrospore germination by the same technique. Parallel observations were

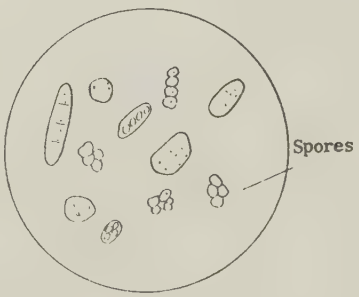

Fig. 1. Yeast spores.

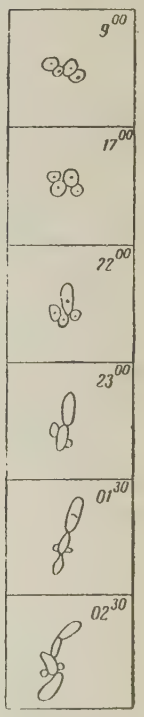

Fig. 2. Spore germination on wort agar in ш-shaped chamber according to Peshkov.

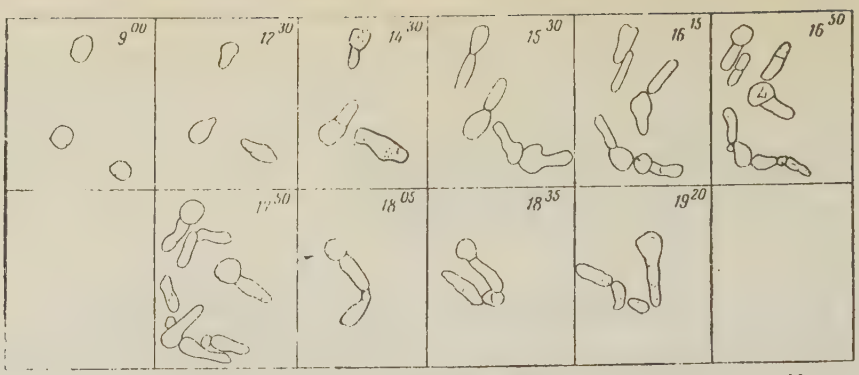

Fig. 3. Nature of arthrospore germination in ш-shaped chamber according to Peshkov.

made on arthrospore germination in the yeast fungi Schizosaccharomyces pombe and Schizosaccharomyces acidodevoratus. After three hours on agar at 35 deg the arthrospores of these yeasts increased in size and elongated and during the fifth hour septa appeared.

In our yeast the arthrospore forms a sprout in three or four hours that in five or six hours becomes separated from the arthrospore by a septum. Later the separated yeast cell reproduces by division.

The germinated arthrospore again forms sprouts from the same area or from the opposite side, which also soon become separated by a septum.

A single arthrospore can produce sprouts three to five times.

It should be noted that in the multiple formation of sprouts there arise several septa that divide the sprouts into cells of various sizes. As a result cylindrical, spherical, and ellipsoidal cells are formed (Figure 3).

The yeast investigated is an active inducer of alcohol fermentation.

It ferments glucose, galactose, sucrose, raffinose, melibiose, maltose, and the dextrins of barley wort. Dextrin fermentation was checked by the extent of fermentation of barley wort with a strength of 15 deg Balling in parallel with cultures of S. vini and S. cerevisiae from the loss of weight in flasks with a Meissel trap during fermentation of 100 ml wort.

The yeast does not ferment lactose and on agar nutrient medium does not utilize glycerol, ethyl alcohol, or acetic, lactic, malic, citric, or tartaric acid.

The behavior toward malic acid was studied on liq uid synthetic medium containing glucose, ammoniur and phosphate salts, 2% yeast autolyzate, and 3–6 malic acid. It was found that the yeast does not utiliz malic acid on this medium.

In view of the fact that the yeast investigated differ from known species of Schizosaccharomyces genu in growth cycle, spore shape, and capacity to ferme galactose and melibiose, it should be classified as new species, Quadrisporomyces adherentes, of th genus Schizosaccharomyces.

SUMMARY

1. A yeast has been isolated which, from its grow cycle, spore morphology, and capacity to ferme galactose and melibiose, can be classified as a ne species of the genus Schizosaccharomyces.

2. The new species has been designated Quadri sporomyces adherentes, genus Schizosaccharomyce from the fact that its spores on release from the ascu do not disperse but stick together in a characteristi arrangement resembling a mulberry.

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DETERMINATION OF THE ISOTOPE EFFECT DURING LABELED CARBON DIOXIDE ASSIMILATION IN PHOTOSYNTHESIS AND CHEMOSYNTHESIS

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It has been established that carbon dioxide containing the radioisotope C^{14} is assimilated more slowly during photosynthesis than the ordinary $C^{12}O_2$. The cause of this retardation in assimilation of $C^{14}O_2$ compared to $C^{12}O_2$ is the somewhat greater molecular weight of the former, resulting in a slower course of the biochemical reactions in which it participates. By reason of the retarded assimilation of $C^{14}O_2$, the isotope composition of carbon of the organic matter, formed during photosynthesis in the presence of $C^{14}O_2$, differs appreciably from that of the initial carbon dioxide. The decrease of C^{14} content in the carbon during conversion from CO_2 to organic matter during photosynthesis, expressed in percent, is termed the "isotope effect". According to Calvin (cited by Rabinowitch, 1956) the magnitude of the isotope effect, determined by the mass spectrometric method, during photosynthesis by unicellular algae in the presence of $C^{14}O_2$ in short-term experiments is about 14%. Steeman Nielsen (1952) calculated the magnitude of the isotope effect during photosynthesis by algae on the basis of experimental data of Van Norman and Brown (1952). According to his calculations, this value is about 5%.

The purpose of the present paper was to determine directly the isotope effect during autotrophic nutrition of Scenedesmus quadricauda algae and of hydrogen-oxidizing bacteria.

The need for obtaining direct data on the isotope effect during photosynthesis and chemosynthesis is necessitated in particular by the fact that at the present time extensive studies are being made on a determination of the organic matter produced in waters, due to photosynthesis by algae and chemosynthesis by bacteria, by means of labeled carbon dioxide containing radioactive C^{14} (Sorokin, 1958, 1959; Steeman Nielsen and Aabye Jensen, 1957). In order to calculate this value correctly from experimental data, it is necessary to know the magnitude of the correction for the degree of "retardation" in $C^{14}O_2$ assimilation, i.e., the isotope effect. The determination of the isotope composition of CO_2 during chemosynthesis in the presence of labeled carbon dioxide also provides an opportunity to check in addition the capacity of the chemosynthesizers to form their body substance exclusively from CO_2 carbon. Solving this problem by use of ordinary bacteriological and chemical techniques leaves certain doubts, since it is difficult to rid the medium and atmosphere completely of traces of organic substances. Hence only a determination of the isotope composition of the carbon of CO_2 and of the organic matter synthesized from it can give a conclusive answer.

METHODS

The isotope effect during autotrophic assimilation of labeled carbon dioxide was determined in our experiments by comparing the specific activity of the CO_2 carbon and that of the organic carbon formed from it during photosynthesis or chemosynthesis.

The experiments on determining the isotope effect in photosynthesis by algae were set up in the following way. Two-liter vessels were filled with medium of the following composition: KH_2PO_4 3 g, $NaNO_3$ 0.03 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, $FeSO_4$ traces; tap water 10 ml; distilled water to 1 liter. After sterilization, the pH of the medium was brought to 6.8 by addition of Na_2CO_3 solution. The medium was inoculated with several drops of Scenedesmus quadricauda algae and to it was added radioactive carbonate solution with total activity (actually counted with counter) of about $2-4 \cdot 10^6$ counts/liter. Vessels were kept in the light for two weeks. The specific activity of the algae organic matter in the culture and that of the CO_2 and bicarbonate in the medium were determined.

For determination of specific activity of CO_2 and bicarbonate in the medium, 20-30 ml of this medium, with content of carbon to be analyzed of not more than

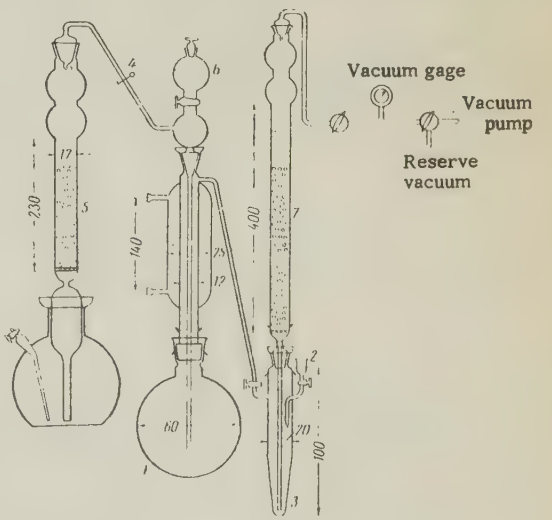

Fig. 1. Diagram of apparatus for carbon determination.

6 mg, was added to flask 1 of the apparatus for semimicrodetermination of carbon, a diagram of which is shown in Figure 1. To side-arm 2 of tube 3 a burette is attached containing 0.05 N alkali purified of CO_2, of which 25 ml is added to the tube. With clamp 4 closed a vacuum of 100–200 mm of mercury is created in the apparatus and its airtightness is checked. By means of the same clamp a small current of air is set up in the apparatus, which on passing through absorbent 5 filled with 20% alkali is freed of CO_2. To flask 1 through funnel 6 is added 5 ml of 5% H_2SO_4 and the liquid in it is brought to boiling; then the burner is removed and the air current through the apparatus increased. The carbon dioxide liberated is carried by the air current to absorbent 7 where it is absorbed by the alkali. Distillation lasts for 5–7 minutes; then the alkali in absorbent 7 is rinsed off with several portions of boiled hot distilled water into a flask with ground stopper of about 130-ml volume with a mark at 100 ml. The liquid volume in the flask is brought exactly to the mark. The liquid is mixed carefully and for determination of carbon radioactivity three samples of exactly 6.7 ml are removed with a Mohr pipette marked accordingly. The samples are transferred to tubes of about 15-ml volume with drawn out ends in which 2 ml 0.1 N KOH purified of CO_2 has been previously placed. Then 0.5 ml 10% $BaCl_2$ is added to the tubes, the tubes are stoppered and kept 8 minutes in a water bath at 60–80 deg. The $BaCO_3$ precipitate is filtered off through a membrane filter in a filter funnel with diameter of filtering area 20 mm made of plexiglass. Residual precipitate on tube and funnel walls is rinsed off with 0.1 N alkali. Membrane filters No. 1–2 of standard dimensions are used for filtration. Filters are boiled prior to use.

The air-dried filters containing $BaCO_3$ precipitate are counted in an end-type counter with window diameter 25 mm. Filters are placed for counting on the support in a groove whose diameter corresponds to the filter diameter (27 mm) and are clamped to the bottom floor of the groove with a lead ring. The support with the filter is adjusted exactly relative to the counter. Distance between filter surface and mica window of the counter is 8 mm. The correction for self-absorption of radiation in the preparation layer was found by means of the curve shown in Figure 2. Data on self-absorption values of $BaCO_3$ precipitates cited in the book of Calvin, Heidelberger et al. (1949) were used to construct this curve. On the ordinate axis are correction coefficient values and on the abscissa the amount of carbon found in the sample analyzed (C_k – see below).

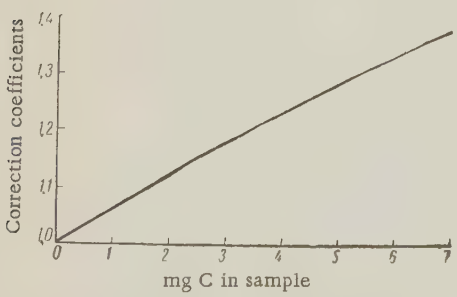

Fig. 2. Curve of corrections for self-absorption of radiation in $BaCO_3$ precipitate layers.

Content of carbon dioxide carbon is analyzed from the residual liquid in the flask (80 ml). For the analysis 3 ml 10% $BaCl_2$ is added to the liquid immediately after removing samples for radioactivity determination, the flask is stoppered and kept in a water bath at 60–80 deg for 5 minutes, and the alkali is back titrated with 0.05 N HCl in the presence of phenolphthalein to a pale pink. A blank determination is run simultaneously. Boiled water is added to flask 1 instead of a sample for analysis. Samples for determination of radioactivity are not removed in the blank determination. Carbon content of CO_2 and bicarbonate in the initial sample (C_k) is calculated from the following formula:

$$C_k = 0{,}3\,(n - n_1 \cdot 1{,}25)\,\text{mg C}$$

where n and n_1 are the volumes in milliliters of 0.05 N HCl used in titrating the blank and the experimental sample, respectively. On the basis of the data obtained the activity of 1 mg carbon was calculated, i.e., the value of its relative specific activity (under the given standard conditions of measurement).

For the determination of specific activity of the carbon in algae cells, the latter were separated from the medium by filtration on membrane filter No. 5 and washed on the filters with 1% H_2SO_4 to remove radioactive carbonate. The algae were transferred from the filter to a combustion tube of 10-ml volume; the tube was attached to the apparatus described above in place of flask 1. Algae were subjected to combustion in this apparatus with 2–3 ml of chrome mixture in the presence of silver sulfate as catalyst with boiling for 3 minutes and passage of a small air current through the apparatus. The chrome mixture was prepared by dissolving chromic anhydride in a mixture consisting of two parts sulfuric and one part phosphoric acid. All the further course of the determination was carried out as described above.

The analyses of specific activity of CO_2 and bicarbonate carbon R and of the organic carbon synthesized from the CO_2 by algae r were conducted under standard conditions and in replicate. Magnitude of the isotopic effect I was calculated from the formula:

$$I = 100\left(1 - \frac{r}{R}\right)\%$$

The isotope effect in assimilation of labeled carbon dioxide during chemosynthesis was determined according to the same basic scheme. To a 10-liter vessel was added 3 liters of medium of the same composition as in the experiments with algae. Medium was inoculated with several drops of a highly active pure culture of hydrogen bacteria isolated from Kuibyshev reservoir water. Labeled carbonate solution was added to the medium, the vessel was closed hermetically, air was removed to a pressure of 500 mm mercury, and hydrogen was introduced. In 20 days the hydrogen bacteria had formed a pellicle on the liquid surface in the vessel. The vessel was removed, the culture shaken and allowed to settle. The precip-

Table. Isotope Effect in Autotrophic Assimilation of $C^{14}O_2$

Assimilation process	Form of carbon	Replicate	Carbon content in sample, mg	Carbon radio-activity, 1000 counts/liter	Carbon specific activity, 1000 counts/mg C	Isotope effect, %
Photosynthesis	Bicarbonate and CO_2	1	1,6	48,3	30,2	6,62
		2	1,53	46,6	30,5	
		3	1,61	48,5	30,2	
		Average	—	—	30,3	
	Organic matter of algae	1	1,41	40,1	28,4	
		2	1,55	43,6	28,2	
		3	1,41	39,6	28,2	
		Average	—	—	23,3	
Chemosynthesis	CO_2 and bicarbonate	1	2,25	81,2	36,1	5,53
		2	2,23	83,5	36,1	
		3	2,7	96,8	35,8	
		4	4,00	144,1	36,0	
		5	3,42	123,5	36,1	
		6	2,87	103,2	35,9	
		Average	—	—	35,98	
	Organic matter of bacteria	1	0,85	28,8	33,9	—
		2	1,17	40,1	34,2	
		3	1,98	67,6	34,1	
		4	2,95	100,6	34,1	
		5	2,78	94,5	33,8	
		6	2,84	96,0	33,8	
		Average	—	—	34,01	

itate, consisting of flocs of bacterial film, was washed by repeated centrifuging in 5% H_2SO_4 solution to remove carbonate. Specific activity of carbon was determined according to the scheme described above in the organic substance of the precipitated bacterial cells and in the CO_2 and bicarbonate of the medium.

EXPERIMENTAL

Typical results of experiments on determining the change in isotope composition of carbon and the isotope effect in photosynthesis by algae in the presence of C^{14}-labeled carbon dioxide are presented in the table. As seen from these data, in the conversion of carbon dioxide carbon during photosynthesis into organic substance its specific activity is reduced appreciably. The magnitude of the isotope effect is about 6.6%. Thus, direct determinations of the isotope effect during photosynthesis by algae give a value somewhat higher than that cited by Steeman Nielsen (1952) and much lower than that found by Calvin et al., in experiments on short-term photosynthesis. According to results of the determinations given in this same table, the isotope effect in chemosynthesis was 5.53%, i.e., a value very close to that of the isotope effect in photosynthesis (6.7%). The somewhat lower value of the isotope effect in chemoautotrophic assimilation of $C^{14}O_2$ may be related to the fact that in photosynthesis the original carbon of the labeled carbon dioxide undergoes numerous transformations before entering the structural elements of the cell, inasmuch as a considerable portion of the primary products of assimilation in photosynthesis goes to form reserve material from which the cell structures are then formed as the result of secondary biochemical processes. Presence of C^{14} in the organic molecules participating in these transformations makes them somewhat heavier and may be the cause of the appearance of an additional isotope effect.

The results of the analyses of change in isotope composition of CO_2 carbon during its assimilation in chemosynthesis demonstrate conclusively the ability of the chemosynthesizers to synthesize all their body substance from CO_2 without the participation of organic compounds dissolved in the water or present in the atmosphere.

Our data on the isotope effect in photosynthesis by algae and in chemosynthesis make it possible to give the values for the respective corrections in calculating the organic matter produced p in bodies of water, due to photo- and chemosynthesis by the vial method with use of C^{14} (see above) from the formula:

$$P = \frac{r \cdot C_k}{R}$$

where r is radioactivity of organic body substance of organisms, R is radioactivity of labeled carbon dioxide added, and C_k is carbon content of the CO_2 bicarbonate in the water. Taking into account the isotope effect, these formula become:

$$P = \frac{r \cdot C_k \cdot 1{,}066}{R}$$ in photosynthesis and

$$P = \frac{r \cdot C_k \cdot 1{,}055}{R}$$ in chemosynthesis.

SUMMARY

The magnitude of the isotope effect (degree of retardation of C^{14} versus C^{12}) was determined during assimilation of labeled carbon dioxide in photosynthesis by Scenedesmus quadricauda and in chemosynthesis by hydrogen bacteria. The determinations were made by comparing the relative specific activities

of the original carbon dioxide and of the organic matter synthesized from it in photo- and chemosynthesis. In photosynthesis the magnitude of the isotope effect was 6.6%, and in chemosynthesis 5.5%. These values can be used as corrections in calculating the organic matter produced in waters, due to photosynthesis and chemosynthesis, as determined by the radiocarbon method.

A convenient method is described for determining specific activity of carbonate and organic carbon.

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ON THE TRANSFORMATION OF FATS BY MICROORGANISMS

II. UNSAPONIFIABLE MATTER IN MICROBIAL DECOMPOSITION OF FATS

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Experiments were described in a previous report on the decomposition of fatty substances by denitrifying bacteria isolated from tertiary petroleums, rocks, and recent organogenic marine silts. The present paper contains the results of an investigation of the unsaponifiable residue from the fat under study.

During alkaline hydrolysis of fat (saponification) the ester bonds are broken and glycerol and potassium salts of fatty acids (soap) go into solution; the unsaponifiable residue can be extracted with ether or other organic solvents. The study of this unsaponifiable residue, consisting of steroids, high molecular alcohols, and hydrocarbons*, is of especial interest from the viewpoint of petroleum genesis.

Unsaponifiable matter was determined by us in combined specimens†, the fatty substances being grouped with respect to degree of change in them and to the origin of the bacterial culture.

The results of the investigation of unsaponifiable substances in the fats under study are given in Table 1. The amount of unsaponifiable matter in the original and control fats is not high (1.43–1.464%), while it is 5.573% in experiments with cultures 1503, 1503-a, and 1503-m isolated from Apsheron petroleum, and under the action of cultures Nos. 3, 4, 6, and 39 from tertiary rock it is 7.583%, i.e., almost 4.6 times more than in the control. In only one case (cultures 1517, 1357, 30/400) was the amount of unsaponifiable matter less than in the control.

According to data of Ravich (1936) the average content of unsaponifiable matter in beef fat is 0.3%. The content of unsaponifiable matter in the case of our original fat was higher than the standard value, and in specimen D exceeded the standard 25-fold. The fact is striking that in three cases the molecular weight of the experimental unsaponifiable residue was considerably greater than in the original and control fats. The high molecular weight coincides with increased content of unsaponifiable matter in the fat. It may be assumed that in these cases there occurred a new formation of certain substances having a higher molecular weight than the original fatty acids‡ and substances in the unsaponifiable residue.

The elementary composition of the substances isolated differs considerably from hydrocarbons of the paraffin series, in which the sum of the percent content of C and H is a figure close to 100. The unsaponifiable residues consist of dissimilar components as indicated by heterogeneity of the material (granules, precipitate) and differences in color of luminescence. The molecular weights** of different parts of the same residue differ considerably among themselves, so that prior to molecular weight determination the substance must be warmed and carefully mixed.

The unsaponifiable residue was divided into three fractions on a column of silica gel of ASK variety with granules 0.5 mm in size. A batch of the material dissolved in petroleum ether was applied to the silica gel and washed successively with dearomatized petroleum ether b.p. 35–50, benzene, and alcohol-benzene. The control for ending the elution of each fraction was the absence of luminescence in the ultraviolet of the solution eluted from the column.

The data on chromatographic analysis and elementary composition of the various fractions are given in Tables 2 and 3 and Figure 1. The petroleum ether fraction was not large in most specimens. It is worthy of note that this fraction was larger in those specimens where the percent carbon was rather high (original fat 76%, specimen F 79.02%). Petroleum ether extracts the most reduced portion of the bitumens, the so-called oily fraction consisting chiefly of hydrocarbons. It must be supposed that in the original fat and in specimen F where the amount of unsaponifiable matter is not great,

*The hydrocarbons present in fats comprise a mixture containing unsaturated hydrocarbons characterized by a high iodine number.

†Fats acted on by cultures 1517, 1357, 30/400 were combined into the single specimen A, by cultures 1503, 1503-a, 1503-m into specimen B, by cultures 552, 585, 17/6, 17/7 into specimen C, by cultures 3, 4, 6, 39 into specimen D, by cultures 9, 36, 58 into specimen E; specimen F was a fat acted on by culture 16-S.

‡The molecular weight of stearic acid is 284.3, of palmitic 256.3, of oleic 282.4.

**Molecular weight and elementary composition were determined in the Bituminological Laboratory of VNIGNI.

Table 1. Results of Analysis of Unsaponifiable Matter Isolated from Beef Fat Acted on by Microorganisms

Specimen	Source of culture	Unsaponifiable matter of fat %	Elementary composition of unsaponifiable matter		Av. molecular wt.	Appearance of material at room temperature	Luminescence in ultraviolet
			C	H			
Original fat (prior to expt.)	—	1.432	76	12	247	Appearance of hardened boiled butter, yellowish solid mass	Tube walls covered with thin layer of material giving light blue light, mass on bottom gives bright yellow-green light
Sterile fat (control)	—	1,649	74,99	12,65	252	Transparent colorless thick liquid with tinge of yellow in which a denser white mass is suspended	Bright white luminescence
A	Tertiary petroleum	1.101	74,32	12,26	242	Solid white mass resembling paraffin	Bright white luminescence with violet tinge
B	Tertiary petroleum	5.573	71,31	11,40	319	Two layers in tube: transparent thick liquid similar to vaseline oil; on bottom nontransparent white mass similar to paraffin	Upper layer—pale blue dull luminescence; lower layer—bright white luminescence
C	Tertiary petroleum	2,071	71,47	11,74	227	Solid white mass resembling paraffin	Uniform light blue luminescence of medium brightness
D	Tertiary rock	7,583	70,53	12,52	331	Colorless thick liquid resembling vaseline oil	Uniform dull bluish gray luminescence
E	Tertiary rock	2.367	75,68	12,42	309	Two layers in tube: transparent thick liquid similar to vaseline oil; on bottom nontransparent white mass similar to paraffin	Upper layer—pale blue dull luminescence; lower layer—bright white luminescence
F	Ground water Devonian	2.465	79.02	12,17	241	Appearance of hardened boiled butter (yellowish solid granular mass)	Tube walls covered with thin layer of material giving light blue light, on bottom yellowish green bright luminescence

Note. No ash in any sample.

Table 2. Group Composition of Unsaponifiable Residue

Specimen	Weight, g	Petroleum ether fraction			Benzene fraction			Alcohol-benzene fraction			Loss
		weight, g	yield, %	appearance	weight, g	yield, %	appearance	weight, g	yield, %	appearance	
Original fat (prior to expt.)	0,0503	0,0026	5,17	—	0,0096	19,09	—	0,0376	74,75	Yellow solid	0,99
Sterile fat (control)	0,2586	0,0020	0,77	—	0,1208	46,71	Light yellow liquid	0,1266	48,96	"	3,56
A	0,1816	0,0018	0,99	—	0,0504	27,75	"	0,1088	59,92	"	11,34
B	0,3028	0,0018	0,60	—	0,2223	73,41	"	0,0736	24,81	"	1,68
C	0,1913	0,0058	3,39	—	0,0178	10,39	"	0,1416	82,66	"	3,56
D	0,2964	—	—	—	0,2342	79,02	Light yellow liquid	0,0603	20,34	"	0,64
E	0,3018	0,0009	0,30	Colorless solid	0,1696	56,20		0,1184	39,23	Orange solid	4,27
F	0,0378	0,0082	21,70	—	0,0070	18,52	"	0,0220	58,20	Yellow solid	1,58

Table 3. Elementary Composition of Benzene and Alcohol-Benzene Fraction of Unsaponifiable Residue

Specimen	Benzene fraction			Alcohol-benzene fraction				
	C	H	S	N	C	H	S	N
Original fat (prior to expt.)	Insufficient material				72.80	11,37	—	0,16
Sterile fat (control)	76,14	12,51	—	0,28	72,66	11,19	—	none
A	79,93	11,34	—	—	74,33	12,33	—	0,56
B	78,63	13,52	—	0,10	71,12	11,59	—	none
C	74,71	11,42	—	0,17	74,50	11,74	—	none
D	74,49	12,33	—	0,99	71,25	10,69	—	0,30
E	78,21	11,75	—	none	72,38	11,72	—	0,33

Table 4. Relation between Molecular Weight Value of Unsaponifiable Residue and Yield of Benzene Fraction

Specimen	Av. mol. wt. of unsaponifiable matter	Benzene fraction yield, %
D	331	79,02
B	319	73,41
E	309	56,20
Sterile fat (control)	252	46,71
Original fat (prior to expt.)	247	19,09
A	242	27,75
F	241	18,52
C	227	10,39

1.4–2.4%, a considerable role is played by hydrocarbons in the composition of these substances.

The benzene fraction, judging from elementary composition, is more reduced than the alcohol-benzene fraction. It usually contains aromatic compounds. The benzene fraction of our specimens varied in the range 10–79%, the highest yield being in specimens with high molecular weight. This relation was quite strictly maintained (Table 4). The small discrepancy in the original fat and in specimen A is most probably due to insufficiently accurate determination of molecular weight as a result of the heterogeneity of the unsaponifiable residue. It is very probable that steroids are concentrated in the benzene fraction, having an aromatic structure, large molecular weight (over 400), and high carbon content.

The alcohol-benzene fraction has the most acid composition; the largest alcohol-benzene fractions are found in those unsaponifiable residues whose yield with respect to total amount of fat is not large—1.4–2.4% (original fat, control, specimens A and F). Judging from the high oxygen content, this fraction must contain chiefly high molecular alcohols and esters.

Study of the infrared absorption spectra of the benzene fractions, carried out by E. B. Proskuryakova, showed that the benzene fractions contained steroids with long side chains of methylene groups, and the alcohol-benzene fractions high molecular alcohols of the steroid group or alicyclic secondary alcohols and esters (higher than propionates) (Figures 2 and 3).

Study of the luminescence spectrum in the ultraviolet of original, control, and treated fats showed no essential changes.

Comparison of the data in Tables 1–4 and the results from spectroscopic studies permits the conclusion that a considerable new formation of steroids occurred in the experiments, which is responsible, mainly, for the increase in the unsaponifiable residue.

Neutral bacterial lipids, from data of Knaysi (1954), contain various amounts of unsaponifiable components, for example cholesterol constitutes 2.5% of the neutral lipids in Lactobacillus acidophilus, wax from Mycobacterium tuberculosis (strain causing avian tuberculosis) contained 10% unsaponifiable matter, wax from Mycobacterium tuberculosis (strain causing bovine tuberculosis) contained 61.03% "unsaponifiable wax".

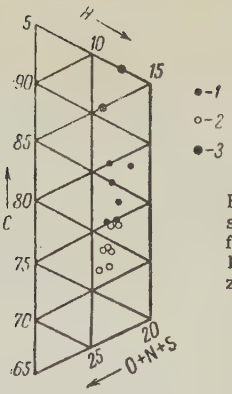

Fig. 1. Triangle of elementary composition of benzene and alcohol-benzene fractions of unsaponifiable residue. 1) benzene fraction; 2) alcohol-benzene fraction; 3) petroleum (average data from A. F. Dobryanskii).

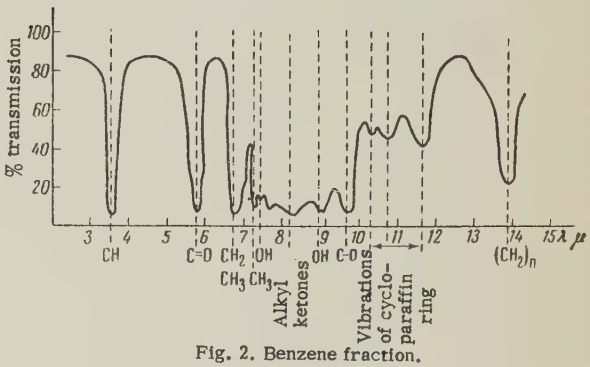

Fig. 2. Benzene fraction.

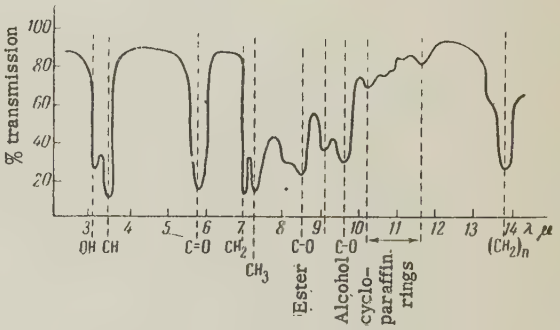

Fig. 3. Alcohol-benzene fraction.

Hecht (1935a, 1935b) rejects the idea that bacteria can only assimilate steroids from the environment; he found steroids in various bacteria even when they were grown on media containing no steroids.

Our claim of new formation of steroids in bacterial cultures is not in contradiction to literature data.

During steroid degradation hydrocarbons are formed rather readily. For example, when cholesterol is heated over palladium-coated carbon the hydrocarbon chrysene (1,2-benzphenanthrene) is obtained, and when heated with selenium the so-called Diehls hydrocarbon (3-methyl-1,2-cyclopentenophenanthrene) is formed. When Zelinskii and Lavrovskii (1041) heated cholesterol in the presence of aluminum chloride, they obtained a petroleum-like substance containing light hydrocarbons of the benzene type.

It may be suggested that bacterial synthesis of steroids from fatty substances in recent marine de-

posits is the source of the formation in more ancient formations of condensed cyclic hydrocarbons.

This suggestion is supported by data of Meinschein (1958), who established by means of mass-spectrometric and spectroscopic studies that the hydrocarbons found in recent marine deposits, sediment rock, and crude petroleum resemble each other and are very similar in structure to certain compounds formed in animal and plant tissues, in particular bile acids, sex hormones, and cholesterol.

SUMMARY

1. During transformation of fats by denitrifying bacberia, the content of unsaponifiable matter increases in the fat acted upon, in some cases 4–6 times compared to control.

2. A new formation of steroids was found in the unsaponifiable residue, which is largely responsible for the accumulation of unsaponifiable components.

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GROWTH OF SOIL ACTINOMYCETES IN MEDIA OF VARYING OSMOTIC PRESSURE

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Many studies on actinomycetes have shown that they are able to grow in soil of considerably lower moisture content than other microorganisms (Krasil'nikov, 1938, 1950, 1958; Novogrudskii, 1946; Teplyakova and Maksimova, 1957; Erikson, 1953; etc.).

According to our observations, actinomycetes were the predominant group of microorganisms in the southern chernozem of Kulunda during the frequent summer droughts when soil moisture fell below the wilting coefficient (Klevenskaya, 1959). Causes of this phenomenon still remain obscure.

The basis of the drought-resistance in actinomycetes evidently lies in their specific morphological and physiological features of structure.

An example of morphological adaptation to unfavorable conditions is the ability of actinomycetes to form a large number of cells resistant to drying (spores), as well as the subdivision of the long mycelial filaments into separate parts, each of which is better protected from drying (Erikson, 1953).

A more important reason for the growth of certain microorganisms in the presence of little moisture is physiological adaptation, expressed in the capacity of the cells to raise the osmotic pressure of the cell solution.

Such physiological adaptation to various conditions of soil moisture, depending on the climate, was observed in soil bacteria by Mishustin and Messineva (1953).

There being no information in the literature on the osmotic pressure in actinomycete cells, we undertook to determine this in cultures isolated from southern chernozem [black soil] and solonetz [saline soil] in the Novosibirsk region.

Table 1. Salt Content and pH of Soil Extracts of Solonetz

Level, cm	Salts, %	pH
In cork-like solonetz		
(0— 3)	0.621	8.58
(3—10)	0.876	8.51
(10—20)	0.711	8.51
(20—30)	0.765	8.7
(30—40)	0.853	8.49
In highly columnar solonetz		
(0— 7)	0.265	7.8
(7—20)	0.201	8.57
(20—30)	0.220	8.7
(30—40)	0.316	8.5

The southern chernozem was located in the northern part of the Kuluda Steppe in an area of insufficient moisture. The average annual precipitation is 266 mm. Soil moisture in summer is extremely low and even in years of most favorable precipitation often falls below the wilting coefficient (9.7% of soil weight).

Total salt content in southern chernozem, from data of Ryabova, is 0.173% of the dry soil weight in the 0–20 cm layer and 0.108% in the 20–40 cm layer.

The solonetz is found in Central Baraba in an area of moderate moisture. Average annual precipitation is 350 mm. Salt content of solonetz is given in Table 1.

The salt composition (milliequivalents per 100 g soil) is: $Ca(HCO_3)_2$, 0.304; $Mg(HCO_3)_2$, 0.324; $NaHCO_3$, 0.304; Na_2SO_4, 2.271; NaCl, 0.323.

METHODS

The determination of osmotic pressure in actinomycete cells is associated with methodological difficulties.

The plasmolytic method of de Vries, usually employed for plant cells and sometimes for bacteria, is not suitable for actinomycetes whose cells are morphologically very complex and grow from the apical part.

The volumetric method of Mishustin (1937), with all its convenience for working with objects such as microbes, cannot be employed in the present case on account of the mycelial structure, the poor wettability of the actinomycete filaments and formation of air spaces between them on centrifuging.

We therefore attempted to determine the osmotic pressure value by an indirect method, based on the ability of actinomycetes to grow on media of varying osmotic pressure.

Seedings were made on Krasil'nikov's liquid medium. Medium I had the following composition: KNO_3 1.0 g, K_2HPO_4 1.0 g, $MgCO_3$ 0.5 g, NaCl 0.2 g, $FeSO_4$ 0.001 g, $CaCO_3$ 0.5 g, tap water 1 liter, pH 7.4.

Sucrose was added as carbon source in 0.005–1.0 M concentration (with 0.001 M differences in sucrose content in the various media) and in 1.0–2.1 M concentration (with 0.1 M differences).

Osmotic pressure of the medium was determined from the respective table in the handbook of Val'ter, Pinevich and Varasova (1957).

Correction for the pressure exerted by the salt components of the medium was calculated from the isotonic coefficient and was 0.8 atm.

A series of media was obtained with osmotic pressure of 0.8–130.9 atm, on which seedings were made to determine the osmotic pressure in the actinomycete cells.

Optimal osmotic pressure was taken as that pressure of the medium at which the best culture growth was observed, the limiting value as the osmotic pressure at which inhibition of growth of the main bulk of cells was observed.

The behavior of actinomycetes towards various salts was checked from the growth intensity of the culture on agarized medium I with addition of various concentrations of salts.

In observing actinomycete growth, note was made of production of mycelium mass, acidification of medium, coloration of aerial and substrate mycelium, liberation of pigments into the medium.

EXPERIMENTAL AND DISCUSSION OF RESULTS

Our analyses made with the actinomycetes most prevalent in southern chernozem and solonetz showed that cultures from the common soil types can grow on media of very low and very high osmotic pressure. Thus, weak growth could be observed on medium containing 0.005 M sugar (osmotic pressure about 0.8 atm).

Actinomycetes grew much better on media with higher sucrose content, 0.1–1.0 M (osmotic pressure 3.4–35.4 atm).

It should be noted that in this case the more active growth of actinomycetes must also be attributed to the increase in energy material in the medium.

An osmotic pressure of 7.4–24.2 atm was found to be optimal for the majority of actinomycetes. Limiting osmotic pressure of the medium, on which actinomycete growth was possible, sometimes reached very high values, for certain species isolated from southern chernozem—88.9 atm and from solonetz—117.4 atm (Table 2).

The capacity of actinomycetes to grow on such media shows that they can develop a pressure within the cell considerably greater than many other plant organisms. Thus, the critical osmotic pressure that certain crops in solonetz of Baraba can tolerate, according to Orlovskii's data, is 12 atm in the presence of sulfate-soda salinity, and 20 atm in the presence of chloride-sulfate salinity (Antipov-Karataev, 1953).

The osmotic pressure of certain soil microorganisms, determined by Mishustin (1937) by the volumetric method, is not over 13.5 atm, in azotobacter 15.3 atm, and in B. mycoides 16.7 atm.

According to data of Panosyan (1948), who used this method, azotobacter isolated from garden soil had an osmotic pressure of 8–14.5 atm, and azotobacter from solonchak [saline soil] 17.3–24.5 atm.

The determinations we made of osmotic pressure in bacteria by the method of seeding on media containing varying sucrose concentration showed that, for example, sharp inhibition of growth was observed in P. aurantiaca at 16 atm, and in B. megaterium at 20 atm.

Higher values for osmotic pressure are generally given for fungi than for bacteria. Lyubimenko (1924) observed growth of mold fungi at 300 atm.

There is reference to the fact that the parasitic fungus Erisiphe graminis has an osmotic pressure of 142 atm (Novogrudskii, 1956).

It should be noted that actinomycetes possess great potentialities for adaptation to conditions unfavorable with respect to osmotic pressure, since they can grow on media of higher osmotic pressure than is observed in soil. The latter, according to data of Antipov-Karataev (1953), is for solonetz of Baraba 2–12 atm, for southern chernozem 2–4 atm.

Despite the fact that actinomycetes can grow at very high osmotic pressure of the medium, they can adapt, like the bacteria, to specific soil conditions. Thus, in actinomycetes isolated from solonetz the optimal growth, pigment formation, acidification of medium,

Table 2. Optimal and Limiting Values of Initial Osmotic Pressure of Medium for Growth of Actinomycetes Isolated from Southern Chernozem and Solonetz

Southern chernozem			Solonetz		
Microorganism	osmotic pressure, atm		Microorganism	osmotic pressure, atm	
	optimal	limiting		optimal	limiting
A. chromogenes	7.4—15.1	66,4	A. chromogenes	7.4—15.1	75,0
A. globisporus	7.4—15.1	88,8	A. globisporus	7.4—24.2	117,4
A. flavus	7.4—24.2	49,2	A. flavus	7.4—24.2	117,4
A. griseus	3.4—15.1	49,2	A. griseus	3,4—24,2	105
A. viridichromogenes	7.4—24.2	88,8	A. flaveolus	7.4—15,1	49,2
A. violaceus	3.4—24.2	88,8	A. candidus	7.4—24,2	117,4
A. fradiae	2.4— 7.4	24,2	A. griscolus	7.4—24,2	88,9
A. coelicolor	2.4— 7.4	35,2	A. syringini	15—35,2	117,4
A. griseoflavus	7.4—15.1	35,2	A. nicrescens	24.2	117.4
A. cylindrosporus	3.4— 7.4	24,2	A. ruber	7,4—49.2	117,4
A. violaceus chromogenes	3.4—15.1	49,9	A. griseoalbus	15,1—35.2	117.4
A. viridis	2.4— 3.4	15,1	A. longisporus ruber	24,2—35.2	117.4
A. globosus	7.4	24,2	A. globosus	7,4—35,2	88,8
A. albus	7.4—24,2	66,4	A. griseorubiginosus	7.4—24,2	88,8
A. halstedii	3,4- 15,1	35,2	A. roseolilacinus	24,2—35.2	88,8
A. rectus bruneus	15,1—24,2	88,9	A. viridis	7,4—15,1	78,0
A. —57	7,4—15,2	24,2	A. cyaneus	7,4—49,2	117,4
A. —19	7,4—24,2	49,2	A.—203	7,4—24,2	117,4
A. —14	15,1—24,2	88,8	A.—204	3,6—24,2	88,9
A. —11	15.1—24,2	88,8	A.—237	2,4— 7,4	47,0
			A. circulatus	7,4—35,2	88,0

Table 3. Limiting Values of Initial Osmotic Pressure and Salt Concentration for Actinomycete Growth

Microorganism	Sucrose		Na_2SO_4		NaCl		$NaHCO_3$	
	concentration, %	osmotic pressure, atm	concentration, %	osmotic pressure, atm	concentration, %	osmotic pressure, atm	concentration, %	osmotic pressure, atm
Southern chernozem								
A. violaceus	56.7	88.8	12.0	43,2	1.5	8.5	0.1	2,4
A. globisporus	56.7	88.8	12.0	43,2	5	28.5	0.1	2,4
A. coelicolor	32.4	35.2	10.0	36.0	5	28.5	0.1	2,4
A. chromogenes	48.6	66.4	3.0	10,8	1.5	8.5	0.01	2,4
A. fradiae	29,16	30.0	5,0	18.0	3.0	17.1	0.1	2,4
A. viridichromogenes	56.7	88.8	3.0	10,8	2.0	11.4	0.01	2,4
A. flavus	40.5	49,2	2.0	7,2	2.0	11.4	0.1	2,4
Solonetz								
A. ruber	64.8	117.4	9.0	32,4	3.0	17.1	0.1	2,4
A. longisporus ruber	64.8	117.4	2.0	7,2	3.0	17.1	0.1	2,4
A. flavus	64.8	117.4	9,0	32,4	5.0	28.5	0.1	2,4
A. globisporus	64.8	117.4	12,0	43,2	7.0	39.9	0.1	2,4
A. viridis	56.7	88.8	9,0	32,4	5.0	28.5	0.01	2,4
A. viridans	53.0	78	5,0	18	5.0	28.5	0,01	2,4
A. globosus	56.7	88,8	7,0	25	5.0	28.5	0.1	2.4
A. griseoloalbus	64.8	117.4	7,0	25	5.0	28.5	0.1	2.4
A. griseorubiginosum	56.7	88,8	3,0	7,2	5.0	28.5	0.1	2.4
A. chromogenes	51.8	75.0	7,0	25,0	5.0	28.5	0.1	2.4

and especially growth inhibition were observed at much higher osmotic pressure of the medium than in actinomycetes from southern chernozem.

Adaptation of soil microorganisms to high osmotic pressure is ordinarily related to a large amount of salt in the soil. We therefore set up special experiments to determine the effect on actinomycete growth of varying concentration of salts and the osmotic pressure produced thereby.

As the experiments showed, the actinomycetes from southern chernozem and solonetz possess high salt resistance (Table 3). All actinomycetes grew well in the presence of 3–7% Na_2SO_4 and 2–3% NaCl.

Certain species, especially those isolated from solonetz, tolerated considerably higher concentrations of these salts (9–12% Na_2SO_4, 5–7% NaCl).

Thus, with regard to salt resistance as well as capacity to grow on media of high osmotic pressure, the actinomycetes can tolerate a much higher salt concentration than that usually observed in soil. The value of the latter, as was indicated above, does not exceed 1% in southern chernozem and solonetz.

Data on the limiting osmotic pressure produced by sucrose and various salts (Table 3) show that the salt toxicity for actinomycetes is related not only to the osmotic pressure increase, but also to chemical properties of the salt. Thus, actinomycete growth was checked at a lower osmotic pressure of the medium in the presence of increased salt concentration than on media containing sucrose.

Sodium sulfate has the least toxicity, sodium chloride the greatest.

Salts that sharply change the pH of the medium are particularly toxic, as for example $NaHCO_3$.

Inhibition of actinomycete growth was observed in our experiments even with addition to the medium of 0.1% soda with an osmotic pressure of 2.4 atm (Table 3).

SUMMARY

1. Actinomycetes are capable of developing high osmotic pressure, which may vary in accordance with environmental conditions.

2. The high osmotic pressure of actinomycetes may be one of the causes for their great prevalence in soils of the southern regions of the USSR, as well as in saline soils.

3. Actinomycetes, isolated from different soils, are distinguished by their capacity to tolerate high salt concentrations. Cultures from solonetz have the greatest resistance to this factor.

4. Toxicity of various salts for actinomycetes is related to their chemical properties as well as to the increase in osmotic pressure.

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ROLE OF THE BIOLOGICAL FACTOR IN TOXICITY OF KOLA PENINSULA SOIL FOR AEROBIC CELLULOSE BACTERIA

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It has been established that toxins, produced by microbial antagonists under certain conditions, may accumulate in soil in appreciable quantity and exert an inhibitory effect on plants and microorganisms. Toxins are produced in soil by certain species of bacteria (Afrikyan, 1954; Krasil'nikov et al., 1955; Rybalkina, 1949), fungi (Mirchink, 1957; Rayner and Neilson-Jones, 1949; Hessayon, 1953), and actinomycetes (Krasil'nikov et al., 1955, Korenyako et al., 1955).

Our studies have shown that aerobic cellulose bacteria are encountered rarely and in small numbers in virgin soils of the Kola Peninsula (Zhukova, 1959a). The extremely slow decomposition of plant residue in these soils is related to the fact that they exert a toxic effect on the growth of cellulose bacteria. Upon cultivation, liming, and manuring of the soil the toxicity decreases but does not disappear (Zhukova, 1959b). The present study was made in order to determine the role of the biological factor in soil toxicity, and the relation of soil toxicity to the number of microbes in it antagonistic to aerobic cellulose bacteria. Species composition of the microbial antagonists was also studied.

EXPERIMENTAL

The antagonist action of bacteria, fungi, and actinomycetes was tested on three species of cellulose bacteria: Vibrio vulgaris, Sorangium cellulosum, and Sporocytophaga myxococcoides. We tested for antagonism to each species of cellulose bacteria 6000 cultures of microorganisms isolated from 35 specimens of cultivated and virgin soil of varying toxicity. In isolating antagonists from soil, dilutions of 1:100, 1:1000, and 1:10,000 were employed. The latter dilution was used only when the soil contained a rich microflora. In seeding soil on meat-peptone, starch-ammonia, and wort agar the 1:1000 dilution was generally used. Seeding of a heated soil suspension on wort-meat agar was made in 1:100 dilution. Two plates were seeded from each soil dilution. Plates were placed in a thermostat at + 25–28 deg for 3–5 days. Colonies were then counted. Plates with the maximal number of single colonies were selected for isolation of antagonists. Transfers were made from each colony to tubes containing medium favorable for antibiotic formation. Colonies of microbes grown on meat-peptone and starch-ammonia agar were transferred to fish agar containing 2% glucose. Colonies grown on wort-meat and on wort agar were respectively seeded on the same agar. In 3–5 days the cultures were tested for antibiotic properties by the method of applying soil clumps. Clumps were placed on the surface of a plate containing Getchinson's agarized medium and filter paper seeded with myxobacteria, and on the surface of Getchinson's starch agar seeded with vibrios. Plates containing clumps were put in a thermostat in a moist chamber. Antibiotic capacity of the tested cultures was determined from the presence of a zone of growth inhibition. Antagonism to vibrios was determined on the next day following seeding, to myxobacteria on the second day.

As a result of the studies a considerable number of antagonistic bacteria (1947 cultures), fungi (1195), and actinomycetes (125) were isolated. Total number of antagonists in the various soils differed (Table 1). On seeding virgin podzol soil on meat peptone and starch-ammonia agar, on which nonsporogenous bacteria mainly grow, the number of antagonists to cellulose bacteria per g soil was 75–360 thousand, on wort-meat agar where sporogenous bacteria grow 11–12 thousand, and on wort agar where fungi predominate 10–49 thousand. In virgin podzol soil the maximal number of microbial antagonists is found in the upper level, A_0, in most cases. Their number decreases with soil depth. At the same time the upper layer of podzol soil is the most toxic (Zhukova, 1959b). The number of antagonists to cellulose bacteria also decreases as a rule with the decrease in total number of microorganisms with soil depth.

On seeding cultivated podzol soil on meat-peptone and starch-ammonia agar the number of antagonists was 45–936 thousand, on wort-meat agar 16–71 thousand, and on wort agar 22–125 thousand. Number of antagonists in cultivated soil depends on soil properties. In soil of low fertility their absolute number is less but, on the contrary, the percentage of total number of soil microflora is greater than in fertile soil. Thus, in iron podzol poor in organic matter the antagonists to Vibrio comprise 9% and to Sporocytophaga 5%, while in humus podzol rich in organic matter they are 4% and 2%, respectively.

To determine the relation of toxicity of soil to its content of microbial antagonists, specimens were selected from each soil type with strong and with weak toxic properties, as well as nontoxic specimens.

Table 1. Number of Microbes, Isolated in Various Media from a Single Soil Sample, Soil Sample, Antagonistic to Aerobic Cellulose Bacteria (in thousands per soil)

Soil	No. of samples	On meat-peptone agar, antagonists to			On wort-meat agar, antagonists to			On starch-ammonia, agar antagonists to			On wort agar, antagonists to		
		Vibrio vulgaris	Sorang. cellul.	Sporocyt. myxoc.	Vibrio vulgaris	Sorang. cellul.	Sporocyt. myxcc.	Vibrio vulgaris	Sorang cellul.	Sporocyt. myxoc.	Vibrio vulgaris.	Sorang. cellul.	Sporocyt. myx.c.
					Virgin								
Podzol	8	130	75	92	11	12	12	220	160	360	33	10	49
					Cultivated								
Iron podzol	5	190	45	115	16	17	18	253	140	430	73	61	72
Humus-iron podzol	5	712	300	408	71	56	52	464	264	400	86	22	62
Humus podzol	6	936	410	466	37	34	35	550	130	265	125	50	95
Upper and transitional marsh	4	250	280	325	21	29	26	300	180	380	86	44	100
Lower marsh	4	385	300	250	28	26	25	320	220	260	95	42	144

Table 2. Number of Antagonists to Cellulose Bacteria on Cultivated Soil of Varying Toxicity

Specimen no.	Soil	Degree of toxicity			Antagonism of cultures isolated to					
		Vibrio vulgaris	Sorangium cellulosum	Sporocytophaga myxococcoides	Vibrio vulgaris		Sorangium cellulosum		Sporocytophaga myxococcoides	
					no. of cultures tested	% antagonists	no. of cultures tested	% antagonists	no. of cultures tested	% antagonists
	Podzol									
902	iron	4	4	4	117	52	120	21	117	55
931	"	4	4	4	69	52	66	40	67	47
734	"	1	1	1	123	20	138	16	139	11
895	"	0	0	1	176	19	179	11	175	22
917	humus-iron	4	4	3	205	46	196	19	181	29
911	"	4	3	3	105	37	106	18	100	38
888	"	1	0	1	134	21	118	15	111	25
898	"	1	1	1	203	45	199	29	204	32
899	humus	4	4	4	202	25	198	8	195	14
861	"	0	2	2	249	39	244	20	239	32
855	Upper marsh	4	4	4	191	44	209	44	210	39
914	" "	4	4	4	132	30	134	17	131	46
857	Transitional marsh	4	4	4	146	34	149	33	148	33
864	" "	0	2	0	154	13	153	5	154	32
900	Lower "	4	4	4	217	48	217	34	214	50
913	" "	2	2	2	94	13	94	10	91	27
860	" "	0	0	0	199	34	207	21	202	14

Symbols: 0—No inhibition, growth of cellulose bacteria over soil plaque normal; 1—growth over plaque less than beyond it; 2—growth only at plaque border; 3—no growth over plaque; 4—no growth over plaque or at some distance from it.

The method of soil plaques was used to determine soil toxicity. It was estimated by the conventional four-point system (Zhukova, 1959b).

It is seen from Table 2 that a correlation is observed between toxicity and number of microbial antagonists in iron podzol of low fertility. Strongly toxic soil contains many antagonists—21–55%, weakly toxic and nontoxic not many—11–22%. In humus and humus-iron podzol the antagonism phenomenon is less clearly marked. In a number of cases the number of antagonists is large in these soils, but toxic action is weak. The humus and humus-iron podzols are considerably richer in decayed matter than iron podzol. Hence their microflora is more numerous and diverse. There are millions of microorganisms per g of humus podzol, of which hundreds of thousands are antagonists. But the effect of the antagonists on toxicity of this soil is much weaker than in iron podzol. This phenomenon is evidently due to the fact that soils richer in decayed matter have greater capacity to inactivate the toxins produced by microorganisms than soils poor in decayed matter (Krasil'nikov, 1951).

There is no distinct correlation between the toxicity of soil to cellulose bacteria and the number of antagonists in it in well-mineralized weakly acid lower marshes or in well-cultivated podzol. But this corre-

Table 3. Number of Antagonists to Certain Species of Cellulose Bacteria in Cultivated Soil

Soil	Antagonism of isolated cultures to					
	Vibrio vulgaris		Sorangium cellulosum		Sporocytophaga myxococcoides	
	no. of cultures tested	% antagonists	no. of cultures tested	% antagonists	no. of cultures tested	% antagonists
Iron-podzol	553	30	575	18	570	28
Humus-iron podzol	882	37	860	28	824	37
Humus podzol	984	33	1050	17	1036	34
Upper marsh	323	38	343	34	341	41
Transitional marsh	300	23	304	18	302	32
Lower marsh	701	32	716	23	700	27

Table 4. Ratio between Different Groups of Microbial Antagonists to Cellulose Bacteria

Soil	Bacteria		Fungi		Actinomycetes	
	no. of cultures tested	% antagonists	no. of cultures tested	% antagonists	no. of cultures tested	% antagonists
Iron podzol	1307	19	296	56	95	11
Humus-iron podzol	1993	30	444	51	129	29
Humus podzol	2154	19	685	43	229	19
Upper marsh	661	23	337	56	9	0
transitional marsh	579	20	241	39	86	17
Lower marsh	1551	22	491	45	75	21

lation is well-marked in soil of upper and transitional marshes; the greatest number of antagonists to cellulose bacteria was found in those soils having a strongly toxic action on these bacteria.

These results warrant the assumption that toxicity of soil for cellulose bacteria is due in large measure to the action of toxins produced by microbial antagonists, whose action is manifested most strongly in soils of low fertility.

The toxic action of soil toward different species of cellulose bacteria is not the same. Most sensitive to it are Vibrio vulgaris and Sporocytophaga myxococcoides, the least, Sorangium cellulosum (Zhukova, 1959b). These observations are in accordance with the manifestation of antagonism toward the different species of cellulose bacteria. From Table 3 it is evident that the number of antagonists to Vibrio and Sporocytophaga is considerably larger than to Sorangium. This apparently accounts for the fact that Sorangium is more prevalent in soil than Vibrio or Sporocytophaga (Zhukova, 1959a).

Investigation of the group composition of the microbial antagonists showed that in all soils, in absolute numbers, there is a considerably greater number of antagonists from among bacteria than from among fungi or actinomycetes. But in relative numbers (in percent) on the contrary, there are twice as many fungal antagonists as bacterial antagonists (Table 4). In all soils 39–56% of the fungi are antagonistic to cellulose bacteria and evidently play a significant part in producing the soil properties toxic to these bacteria. Neilson-Jones (1941) noted that in toxic soils of Wareham the number of fungi and bacteria is low and there are not many species. Later a number of workers showed that in these soils three Penicillium species predominate, which disturb the microbial balance in the soil by liberating antibiotics (Brian, Hemming and Gowan, 1945).

Our observations are in accord with these data. In strongly toxic soil the species composition of the fungal antagonists is limited and is represented chiefly by fungi of genus Penicillium or Trichoderma (Table 5).

Thus, from humus-iron podzol (No. 884) of high toxicity 36 fungi were isolated and identified, of which 33 cultures belonged to the Monoverticillata group of genus Penicillium. In a lower marsh (No. 900) of high toxicity the antagonists were fungi of genus Penicillium and Trichoderma. In this soil the fungus Trichoderma album was most prevalent. Of 42 cultures isolated 31 belonged to this species, which has high antibacterial activity. In the nontoxic and weakly toxic soils the species composition of fungal antagonists is diverse. Thus, from a transitional marsh with weakly toxic properties (No. 864) 26 cultures were isolated of which 6 belonged to the Asymmetrica group of Penicillium, 4 to Trichoderma lignorum, 4 to Trichoderma album, 2 to Aspergillus genus, 3 to Stemphylium genus, and 7 to Acrostalagmus genus. Consequently it may be supposed that the interaction of fungi among themselves reduces their toxic effect toward cellulose bacteria.

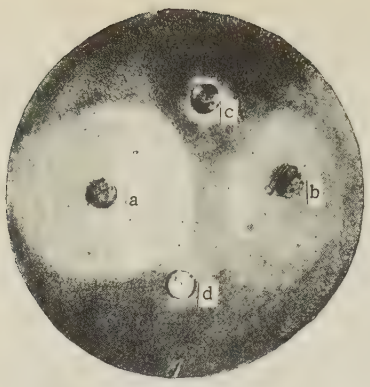

Fig. 1. Antibiotic activity of Penicillium strains on Sporocytophaga myxococcoides. a—Zone of no growth 12-15 mm around P. cyclopium No. 2183; b—zone of no growth 5-7 mm around strain No. 3668; c—zone of inhibited growth 1-2 mm around cyclopium strain No. 1445; d—no zone around strain No. 3239.

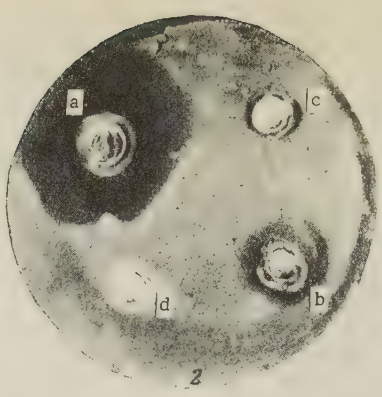

Fig. 2. Antibiotic activity of Penicillium strains on Vibrio vulgaris. a—Zone of no growth 10-13 mm around P. cyclopium No. 2183; b—zone of inhibited growth 3-4 mm around strain No. 3868; c—zone of inhibited growth 1-2 mm around strain No. 1445; d—no zone around strain No. 3239.

Table 5. Predominant Forms of Fungi Antagonistic to Cellulose Bacteria

Specimen no.	Soil	Deg. toxicity of			No. of cultures identified	Genus									
						Penicillium group			Trichoderma species						
		Vibrio vulgaris	Sorang cellulosum	Sporocito. phaga myxococcoides		Monoverticillata	Biverticillata	Asymmetrica	Lignorum	Album	Koningii	Aspergillus	Fomaia	Fusarium	Stemphylium	Acrosta fagmus
902	Iron podzol	4	4	4	24	25*	—	—	58	—	—	4	—	4	8	—
884	Humus-iron podzol	4	4	4	36	91	5	—	—	—	2	—	—	—	—	—
899	Humus podzol	4	4	4	19	47	10	31	—	—	—	—	5	5	—	—
855	Upper marsh	4	4	4	46	30	2	2	54	8	—	—	—	1	—	—
914	Upper marsh	4	4	4	54	7	—	5	20	—	59	1	—	1	—	
900	Lower marsh	4	4	4	42	—	—	9	—	71	16	—	—	—	—	—
890	Humus-iron podzol	0	0	0	13	—	7	—	46	7	7	—	15	15		—
864	Transitional marsh	0	1	0	26	—	—	23	15	15	—	7	—	—	11	26
856	Lower marsh	0	0	0	23	—	8	39	39	—	—	—	—	—	13	—

*In percent of number of cultures identified.

Table 6. Change in Soil Toxicity for Cellulose Bacteria on Addition of Antagonists to Nonsterile Soil

Antagonist added		Right after seeding			After 4 days			After 18 days		
		degree of toxicity for								
		Vibrio vulgaris	Sorangium cellulosum	Sporcytophaga myxococcoides	Vibrio vulgaris	Sorangium cellulosum	Sporcytophaga myxococcoides	Vibrio vulgaris	Sorangium cellulosum	Sporcytophaga myxococcoides
		Iron podzol								
Control (uninoculated soil)		0	0	0	0	0	0	0	0	0
Penicillium	4528	0	0	0	0	0	2	0	0	1
»	902	0	0	0	0	1	2	0	0	0
Trichoderma	553	0	0	0	1	1	2	1	0	1
B. mesentericus	3271	0	0	0	2	2	2	0	1	1
		Humus-iron podzol								
Control (uninoculated soil)		0	0	1	0	0	1	0	0	1
Penicillium	4528	0	0	1	0	1	1	0	0	2
»	902	0	0	1	0	1	1	0	0	2
Trichoderma	553	0	0	1	0	1	1	0	1	1
B. mesentericus	3271	0	0	1	1	0	1	0	2	2

Symbols—see Table 2

Table 7. Change in Soil Toxicity for Cellulose Bacteria on Addition of Antagonists to Nonsterile Soil with Sucrose

Antagonist added	Right after seeding			After 4 days			After 18 days		
	degree of toxicity for								
	Vibrio vulgaris	Sorangium cellulosum	Sporocytophaga myxococcoides	Vibrio vulgaris	Sorangium cellulosum	Sporocytophaga myxococcoides	Vibrio vulgaris	Sorangium cellulosum	Sporocytophaga myxococcoides
Iron podzol									
Control (uninoculated, unfertilized soil)	0	0	0	0	0	0	0	0	0
Control (uninoculated, fertilized soil)	0	0	1	0	2	2	2	2	2
Penicillium 4528	0	0	1	0	2	2	0	4	2
» 902	0	0	1	0	2	2	0	1	1
Trichoderma 553	0	0	1	3	2	3	1	4	2
B. mesentericus 3271	0	0	1	3	2	3	0	0	1
Humus-iron podzol									
Control (uninoculated, unfertilized soil)	0	0	1	0	0	1	0	0	1
Control (uninoculated, fertilized soil)	0	0	1	1	2	2	0	2	2
Penicillium 4528	0	0	1	1	2	2	0	1	2
» 902	0	0	1	0	2	2	0	1	1
Trichoderma 553	0	0	1	0	1	2	0	1	2
B. mesentericus 3271	0	0	1	0	2	1	0	0	2

As Mishustin and others have noted, a reduction in cellulose bacteria is observed in soil where there is increased growth of saprophytic putrefying bacteria (Mishustin and Timofeeva, 1944; Teplyakova, 1955). B. herbicola and bacteria of Pseudomonas genus are antagonists from among the nonsporogenous putrefying bacteria; of the spore-formers, bacteria from the group B. mesentericus, B. subtilis, B. pumilis, and others are antagonists. Most of the bacterial antagonists inhibit but do not entirely suppress growth of cellulose bacteria. The B. mesentericus group has an especially strong bacteriostatic action. A number of fungi and actinomycetes have a bactericidal action on cellulose bacteria. These include Penicillium cyclopium (Figures 1, 2) and actinomycetes from the group A. lavendulae, A. fluorescens, A. rimosus.

In order to determine whether microbial antagonists can produce their characteristic antibiotics directly in the soil, weakly toxic and nontoxic podzol soils were artificially inoculated with antagonists; fungi of genus Penicillium and Trichoderma and sporogenous bacteria of the B. mesentericus group. The experiments were set up with nonsterile soils with and without sucrose, which is not toxic to myxobacteria and vibrios even when the content in medium is 10% (Imshenetskii, 1941). Sucrose was added to soil in our experiments in the amount of 1% of soil weight. Soil was inoculated with an aqueous suspension of fungus spores or suspension of spore-forming bacteria. The control was uninoculated soil. Soil was moistened to 60% of full moisture capacity. In order to maintain constant moisture the plates containing soil were kept in a moist temperature at + 27 deg. The toxicity of the soil for cellulose bacteria was determined 4, 8, and 18 days following its inoculation with the microbial antagonists (Tables 6, 7).

As seen from Table 6, upon inoculating nonsterile and unfertilized podzol soils with antagonistic fungi and sporogenous bacteria, their toxicity for cellulose bacteria increases in most cases. Upon inoculating nontoxic iron podzol with antagonists, the increase in toxicity is greater than upon inoculating humus-iron podzol with the same antagonists.

In soil containing sucrose the same microorganisms form more toxic substances than in soil without sucrose (Table 7). After four days of incubation, however, even in the uninoculated soil containing sucrose an increased toxicity for cellulose bacteria was observed. But this did not occur in uninoculated, unfertilized soil. This phenomenon is explained by the fact that after addition of sucrose to nonsterile soil, an active growth of the microflora begins, including fungi that inhibit growth of cellulose bacteria. Thus, there were 17 thousand fungi per g iron podzol. After incubation of this soil for 18 days there was practically no change in number, while in the same soil containing sucrose the number of fungi increased four times. The growth of fungi and their production of antibiotics is then apparently the reason for the appearance of substances toxic to aerobic cellulose bacteria in nonsterile, uninoculated soil containing sucrose.

I express deep gratitude to Professor N. A. Krasil'nikov for the guidance of this work.

SUMMARY

1. Podzol and peat-marsh soils of the Kola Peninsula are toxic to aerobic cellulose bacteria. Microbes that inhibit or completely suppress growth of cellulose bacteria were found in these soils in considerable numbers.

2. The greatest number of microbes antagonistic to cellulose bacteria, in iron podzol of low fertility and in soil of upper and transitional marshes, was found in those soils with high toxicity. This warrants the

assumption that antagonists play an important role in producing soil toxicity.

3. Antagonism toward cellulose bacteria and other soil microorganisms is less apparent in well-cultivated soils rich in decayed matter. There is no correlation between toxicity and number of antagonists in these soils; this is apparently due to the great capacity of the soils to inactivate toxins.

4. Soil contains considerably more microbes antagonistic to Vibrio vulgaris and Sporocytophaga myxococcoides than to Sorangium cellulosum.

5. Fungi and sporogenous bacteria are the principal antagonists to cellulose bacteria. In highly toxic soils the species composition of fungal antagonists is limited and is represented chiefly by Penicillium and Trichoderma.

6. The microorganisms antagonistic to cellulose bacteria are able to form toxic substances in nonsterile podzol soil, unfertilized or fertilized with sucrose.

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EFFECT OF MOISTURE AND LOW TEMPERATURE ON NUMBER OF BACTERIA IN SOIL

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Many studies have been made of the seasonal variations in quantitative composition of the bacterial population in soil.

Most of these studies, however, are restricted to growth dynamics of the soil bacteria only during the vegetative period. There are considerably fewer studies on growth dynamics of soil bacteria during the winter. Certain investigators (Conn, 1912; Levinskaya and Mamicheva, 1956; and others) note that increased bacterial content in soil was observed in winter. The data of Butkevich (1958) and Chistyakov and Noskova (1938) on bacterial growth at low temperature are of interest.

In the winter of 1953/54 and summer of 1954 we made several determinations of the content in various soils of azotobacter and bacteria that grow on MPA. These preliminary analyses showed a high bacterial content in severely frozen soil. Regular analyses of soil from two sections of different soil regions 2 × 2 m in size were made from the beginning of the winter of 1954/55.

Soil from the first section was turfy gley, saturated, on sand; that of the other section was typical turfy carbonate soil, loam on limestone. Samples for analysis were taken in series from one site at three levels, 5, 10, and 25 cm.

Eight physiological groups of soil bacteria were found to be present. In analyzing the putrefying bacteria, several other media in addition to MPA were tested. We chose a new medium of the following composition: peptone 10.0 g; Na_2HPO_4 2.0 g; $MgSO_4$ 0.5 g; $FeCl_3$ 0.005 g; $CaCO_3$ 0.1 g; KH_2PO_4 0.1 g; agar 20 g; water 1 liter. On this [Fedorov's medium; Clostridium pasteurianum, butyric acid, nitrifying] but their number was always greater. Azotobacter and oligonitrophils were estimated on Fedorov's medium; Clostridium pasteurianum, butyric acid, nitrifying, denitrifying, and aerobic cellulose-decomposing bacteria on the media and by the technique approved by the All-Union Conference of Soil Microbiologists in 1953. The first dilution was shaken on a shaker for 10 minutes.

A total of 151 analyses were made in the relatively severe winter of 1954/55, which gave quite interesting results. Although all the soils investigated were frozen even at the bottom level, the number of bacteria in general did not decrease, but increased. However, the objection may be raised regarding the results obtained that, although samples from small and carefully selected sections were analyzed, nevertheless they might be insufficiently uniform to make conclusions concerning the bacterial dynamics. Another series of experiments was therefore set up in August 1956.

In these a biometer was built—a bottomless wooden box 1.8 × 1.75 m in size, separated below from the lower soil levels by a 10-cm layer of chips. On top of the chip layer was placed a 45-cm layer of turfy gley soil that had been carefully mixed and passed through a sieve. Samples for analysis were taken in series from two sites and two levels and were analyzed separately. The parallel samples analyzed in the course of a year and a half gave very close results, being almost completely similar in many cases. Analyses were made every two weeks on the average. Next to the biometer, a plant container with 8.15 kg of soil was dug into the ground and to it was added 3.5 g krilium. Samples for analysis were taken from the top layer in the container.

In order to avoid as far as possible the effect of outside factors, weed sprouts were destroyed in the biometer as soon as they appeared. Hence it is difficult to raise the objection in the present case of non-uniformity in the analyzed soil.

The analytical results are given in Table 1.

The data presented for the 1956-1958 analyses confirm the results of the 1954/55 analyses. In periods of high percent of moisture and low temperature, which was in most cases below 0 deg, i.e., in frozen soil, a higher content of putrefying bacteria and oligonitrophils is noted. Although so high a content was not noted in the other groups of microorganisms studied, the low temperature did not materially reduce their number.

Experiments were carried out early in 1957 on the multiplication of soil bacteria directly in frozen soil. The first of these experiments gave such surprising results that it was necessary to repeat and modify them several times in order to be convinced of their reliability.

In these laboratory experiments soil from our biometer was used, to which certain nutrients had been added: sugar, peptone, finely ground filter paper, etc. An analysis of the initial soil with added nutrients was made, then the soil was placed in the icebox in which a definite temperature was maintained, and microbiological analyses of the soil were made periodically.

Seven series of such experiments were conducted in the course of two years.

The results are given in Table 2.

Table 1. Average Analytical Data on Soil from Biometer with Varying Soil Temperature and Moisture in 1956–1958

Expt. no.	Period	No. of analyses	Av. soil temperature, deg C	Soil moisture, %	No. of bacteria, thousands per g soil						
					putrefying		oligonitrophils	denitrifying	nitrifying	cellulose-decomposing	azotobacter
					total number	Bac. mycoides					
	I level (5 cm)										
I	3.8.1956 to 21.2.57	18	+ 2.3	29.0	3136	28	67.0	296	51.0	16.0	0.304
II	28.2.1957 » 7.3.57	20	— 1.1	35.4	4879	30	74.0	85	51.0	2.0	0.353
III	5.4.1957 » 12.7.57	20	+12.5	26.5	2323	20	37.0	122	22.0	5.0	0.433
IV	9.8.1957 » 28.12.57	22	+ 6.7	25.8	2414	17	49.0	364	19.0	7.0	0.359
V	10.1.1958 » 11.4.58	20	— 1.1	41.2	3222	17	34.0	187	15.0	7.0	0.287
	II level (25 cm)										
I	3.8.1956 to 31.10.56	14	+ 6.3	28.0	3640	26	75.0	231	56.0	10.0	0.466
II	13.2.1957 » 26.4.57	28	+ 0.9	32.8	5044	31	70.0	132	37.0	2.0	0.379
III	10.5.1957 » 12.7.57	16	+12.1	27.2	2512	21	36.0	64	15.0	4.0	0.581
IV	9.8.1957 » 21.2.58	34	+ 5.3	26.1	2807	17	44.0	264	20.0	8.7	0.360
V	6.3.1958 » 28.4.58	10	0	38.5	4343	18	59.0	204	15.0	4.7	0.248
	Top layer from plant container (with krilium)										
I	3.8.1956 to 21.2.57	8	+ 2.3	29.3	2266	21	55.0	238	25.7	4.6	0.145
II	28.2.1957 » 26.4.57	10	— 1.1	41.2	2751	23	45.2	85	11.4	0.9	0.205
III	10.5.1957 » 25.10.57	16	+12.0	26.4	2682	19	56.2	104	18.7	16.6	0.267
	Average for all data										
	In periods with moisture below 30%	148	+ 7.9	26.9	2714	20	49.0	218	27.0	8.3	0.374
	In periods with moisture above 30%	88	— 0.4	36.9	4252	25	59.0	138	30.0	3.4	0.316

Table 2. Changes in Number of Certain Bacterial Groups with Addition of Nutrients to Soil and Maintenance at Low Temperature

Expt. series	Temperature fluctuation in deg C	Moisture, %	Duration of maintenance, days	Nutrients added, % of soil wt.				No. of bacteria in thousands per g dry soil			
				peptone	sugar	paper + KNO	Getchinson medium	putrefying	oligonitrophils	denitrifying	aerobic cellulose-decomposing
I	—1.5 to —2.3	13.0	0	—	—	—	—	3146	26.5	10	0.390
			6	—	—	1	—	—	—	—	2.900
			28	1	—	—	—	52 359	—	—	—
II	—1.5 to —2.3	23.0	0	—	—	—	—	2 821	15	7.4	—
			8	—	2	—	—	15 959	153	7.5	—
			8	2	—	—	—	53 968	18	31	—
			28	—	2	—	—	126 900	63	76	—
			28	2	—	—	—	189 800	51	1392	—
III	—1.5 to —2.3	26.0	0	—	—	—	—	8777	—	—	2.040
			13	—	—	2+0.8	—	—	—	—	15.000
			150	2	—	—	—	72 000	—	—	—
IV	—1.5 to —2.3	30.0	0	—	—	—	—	6773	—	—	1.600
			13	—	—	2+0.3	—	—	—	—	10.100
			55	—	2	—	—	93 347	—	—	
V	—3.2 to —4.8	20.0	0	—	—	—	—	482	26	6.4	0.640
			33	—	2	—	—	152 000	445	1392	8.800
			33	2	—	—	—	9 924	50	31	3.100
			51	2	—	—	—	487 000	50	312	1.500
VI	—1.5 to —2.3	33.0	0	—	—	—	—	9595	106	544	2.200
			12	—	—	—	2	18 232	89	1751	17.500
			34	—	—	—	2	85 624	118	1682	10703.000
	—3.8 to —6.0	23.0	26	—	—	—	2	8434	74	1054	3.000

Table 2. (continued)

Expt. series	Temperature fluctuation in deg C	Moisture, %	Duration of maintenance, days	Nutrients added, % of soil wt.				No. of bacteria in thousands per g dry soil			
				peptone	sugar	paper + KNO	Getchinson medium	putrefying	oligonitrophils	denitrifying	aerobic cellulose-decomposing
VII	−1.1 to −2.3	7.0	0	—	—	—	—	1620	14	65	0.270
			60	—	2	—	—	7869	25	268	0.050
		23.0	80	—	2	—	—	267 016	196	8	3.300
	−4.0 to −5.3	7.0	60	—	2	—	—	2447	26	64	0.080
		23.0	60	—	2	—	—	10 052	38	79	0.330

Table 2 shows that various groups of soil bacteria are able to utilize nutrients and actively multiply in frozen soil at constant temperature considerably below zero. They multiply more actively in those soils where water content (in the present case ice content is more accurate) is rather high (data from experiment series VII, where number of putrefying bacteria in 60 days at 7% moisture increased insignificantly, but in 80 days at 23% moisture increased more than a hundred times).

The results of the laboratory experiments supplement the results of analysis of soil from the biometer and from field sections and confirm their reliability.

Consequently, microbiological processes in frozen soil in winter are not extinguished, but may proceed quite actively in many cases.

SUMMARY

1. Certain soil bacteria (putrefying and oligonitrophil) may reach a maximal number in frozen soil in winter, under the climatic conditions in the northern temperate zone.

2. Many groups of soil bacteria, under laboratory conditions as well, can multiply vigorously in frozen soil at −1.5 to −5.0 deg when nutrients are added to the soil.

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RESISTANCE OF CERTAIN SPECIES OF SOIL MICROORGANISMS TO ANABASIN

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In studying the microflora in takyr soil of the ancient Prisarykamyshskaya Delta of the Amu-Darya under various plant associations, we found a certain specificity in composition of the microbial population in cultivated soil and deposits of varying age (Konobeeva, 1956). From irrigated takyr soil under cotton, just as in takyr soil of the stage of the Alhagi pseudalhagi association, and from the rhizosphere of plants growing in this soil the following bacteria were isolated: Azotobacter, Clostridium pasteurianum, B. mycoides, B. megaterium, B. mesentericus, B. idosus, B. cereus, lactic acid bacteria, pigmented bacteria of Pseudomonas genus, and others.

B. megaterium was most prevalent in soil under cotton and in the cotton-plant rhizosphere.

Takyr soil of the stage of the Anabasis aphylla association and the rhizosphere of Anabasis aphylla were distinguished by an extreme poverty of microflora compared with the preceding and succeeding deposits, despite the relatively short interval of time separating these soils from the period of former irrigation. Here such microorganisms as Azotobacter, Clostridium pasteurianum, B. mycoides, B. megaterium, and others were absent.

At the same time the microflora of takyr soil of the stage of the Anabasis aphylla association was characterized by the presence of anaerobic cellulose decomposers, actinomycetes, and a high content of sporogenous microorganisms, the dominant form of which was B. mesentericus.

Anabasis aphylla is known to be a source of the alkaloid anabasin, which is present in the green stems, stalks, and roots of the plant. According to data of Sadykov (1956), the Anabasis aphylla growing in the Kunya-Urgench district of Tashauzskaya oblast contains 3.5% alkaloids of which 2.4% is anabasin.

Upon the withering of the plant every year and the leaching of it by atmospheric precipitation, anabasin gets into the soil.

We suggested the hypothesis that the anabasin, being a very stable alkaloid, might be inhibitory to the growth of certain groups and species of microorganisms.

In order to check this hypothesis, we set up experiments on the effect of pure anabasin on pure cultures of microbes isolated from the rhizosphere of the cotton plant and Alhagi pseudalhagi, i.e., from beneath plant formations preceding the Anabasis aphylla association. We tested Azotobacter, Clostridium pasteurianum, B. mycoides, B. megaterium, lactic acid bacteria, as well as cultures specific for the rhizosphere of the Anabasis aphylla association—B. mesentericus and actinomycetes.

Anabasin dilutions of 1:10, 1:100, 1:1000 in sterile distilled water were employed. The sensitivity to anabasin of Azotobacter, B. mycoides, B. megaterium, B. mesentericus, and one actinomycete strain was tested on solid nutrient media. The technique used for testing antibiotics (the groove method) was used. For this a Petri dish was filled with a thick layer of MPA mixed with wort agar. After the agar hardened a strip of it 1 cm wide was cut out along the dish diameter, and here anabasin solution was added. The culture was seeded in a streak from the two sides of the groove and perpendicular to it. Growth of B. mycoides, B. megaterium, B. mesentericus were examined after 18, 24, and 48 hours, and the actinomycete in 3–5 days. Results were determined from presence and intensity of culture growth.

For Clostridium pasteurianum and lactic acid bacteria, appropriate selective nutrient media were employed containing anabasin in an amount such that on addition of 1 ml of a 24-hour culture of the tested microorganisms, concentrations of 1:10, 1:100, and 1:1000 were obtained in the solution.

Control cultures of lactic acid bacteria and Clostridium pasteurianum were grown in the liquid selective medium. Results were estimated from growth intensity on the fifth day, by microscopic examination, and by subsequent seeding on the selective medium.

The results of testing pure cultures grown on MPA medium mixed with wort agar and containing anabasin are shown in the table.

Appreciable growth of actinomycetes on medium containing anabasin was observed in three days, on the fifth day the greater part of the plate surface was overgrown. The actinomycetes developed vigorously not only on the agar surface, but even in the grooves treated with anabasin in all the tested concentrations.

Experiments with cultures on liquid media give the following results. No growth of Clostridium pasteurianum was noted at any anabasin concentration tested. Nor did lactic acid bacteria grow at any anabasin concentration tested. Cells of lactic acid bacteria were found on microscopic examination to be greatly deformed.

Sensitivity of azotobacter to anabasin was tested by the following technique. Several drops of anabasin solution of proper concentration was added to a 1-g batch of soil until a dough-like consistency was obtained, and then soil clumps were prepared from it

Table. Effect of Anabasin in Varying Concentration on Growth of Microorganisms on Solid Media

Microorganism	Anabasin concentration								
	1 : 10			1 : 100			1 : 1000		
	18 hr	24 hr	48 hr	18 hr	24 hr	48 hr	18 hr	24 hr	48 hr
Bac. mycoides	no growth			no growth	growth very weak		no growth	growth very weak	
Bac. megaterium	no growth	growth 1 cm from groove		no growth	growth beginning 0.5 cm from groove		no growth	growth from groove, but very weak in 1–1.5 cm zone	
Bac. mesentericus	no growth		growth abundant 0.4 cm from groove	no growth	growth beginning at edge of groove		no growth	abundant growth over whole plate	

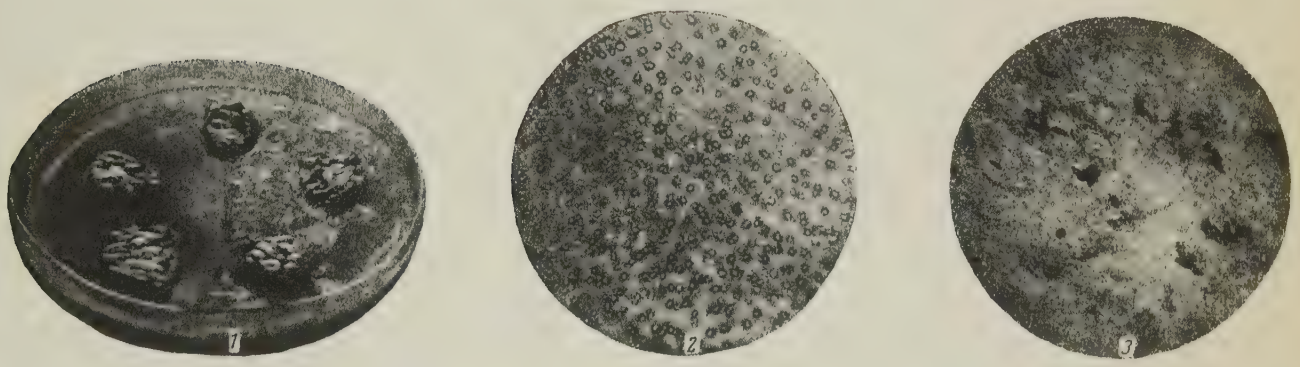

Fig. 1. Growth of azotobacter around clumps of takyr soil containing anabasin. At left—sterile zone around soil clumps with pure anabasin and 1 : 10 solution. At right—zone overgrown with azotobacter (control clumps and those with anabasin 1 : 100 and 1 : 1000).

Fig. 2. Azotobacter growing around soil clumps with anabasin 1 : 100 and 1 : 1000 (Mag. 900x).

Fig. 3. Azotobacter and satellite from zone of contact with sterile area (Mag. 900x).

Petri dishes containing agarized Ashby medium were seeded with a 4-hour culture of azotobacter. Then soil clumps were placed on the seeded plate: two clumps moistened with sterile distilled water (control), two clumps with pure anabasin, and two with anabasin in different concentration.

Experiments were done in duplicate. Plates were examined after 18, 24, and 48 hours of maintenance in the thermostat.

Within 18 hours there was formed around the clumps with anabasin concentration 1:100 and 1:1000 a sterile zone with uniform width of about 0.4 cm, which was retained for six hours longer and on the second day was overgrown. Around clumps with pure anabasin and with concentration 1:10 was a large sterile zone of 20 mm radius in the first plate and 15 mm in the duplicate (Figure 1), which was retained for one month (duration of experiment). A control seeding from the edge of the sterile zone to solid and liquid Ashby medium gave no azotobacter growth. No azotobacter was present in a smear from the sterile zone, but the satellite organism was observed. Smears from the very edge of the sterile zone contained large numbers of small bacilli of the azotobacter satellite; azotobacter cells were greatly deformed and appeared as dark amorphous masses (Figures 2 and 3).

Sukhorukov and Malysheva (1956), who tested the action of anabasin sulfate on the plant, note that it increases permeability of cell plasm, increases activity of oxidative enzymes, decreases reducing capacity, causes layering off of lipoids and death. The same thing is probably true for azotobacter cells. The satellite organism accompanying azotobacter was found to be more resistant to anabasin.

Smears from the culture of azotobacter grown around the control clump contained normal well-developed azotobacter cells and solitary cells of the satellite organism.

SUMMARY

1. Anabasin in 1:10 concentration is bactericidal to azotobacter, B. mycoides, B. megaterium, B. mesentericus, Clostridium pasteurianum, and lactic acid bacteria.

No abasin concentration tested exerted a bactericidal effect on actinomycetes.

2. Anabasin 1:100 is bacteriostatic to azotobacter, B. mycoides, and B. megaterium, but does not affect B. mesentericus.

3. Anabasin 1:1000 is bactericidal only to lactic acid bacteria and Clostridium pasteurianum, and slightly inhibits growth and development of azotobacter, B. mycoides, and B. megaterium. B. mesentericus grows well at this concentration.

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EFFECT OF REPEATED APPLICATIONS OF HERBICIDES ON SOIL MICROFLORA

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Various herbicides affect soil microflora in different ways. Preparations TKhA, PKhF, IFK (Aldrich, 1954) and chlorates in specific dosages had a toxic effect. Usually the following dosages of herbicides were used per hectare (in kg): TKhA 12–20, PKhF 8–10, IFK 10–15, and chlorates 300–500. A solution of maleic hydrazide in concentration between 1:100 and 1:10,000 did not suppress the vital activity of the soil microorganisms (Webber, 1956). The majority of investigations were devoted to the presently most important herbicide, the preparation 2.4-D, which is usually applied in dosages of 1–2 kg/ha. It was noted (Aldrich, 1954, Repp, 1956, Sokolov, 1956, Tsitovich, 1954) that 2.4-D did not have a negative effect on microflora. In Dekatov's opinion (1955-1958), the activity of the beneficial microorganisms is intensified as a result of applying the herbicide in a dosage of 1–3 kg/ha. Algren, et al., (1953) indicated that 2.4-D in the commonly applied dosage severly inhibits the growth of aerobic microorganisms and does not substantially affect the anaerobic. At the same time it can stimulate the development of many facultative anaerobes. The data in the aforementioned studies apparently refer to a single application of herbicides.

In 1956–1958 we investigated the application of herbicides for combating weeds in field-protecting forest belts during the first years after planting, mainly in rows of woody seedlings. Since the chemical treatment in this case was repeated numerous times in the course of several years, it was interesting to find out if the repeated application of herbicides had a severely negative effect on soil microorganisms.

The studies were conducted in a forest belt planted in April, 1957. The soil was common chernozem.

Table 1. Herbicides Used in the Experiment, Their Dosage and Application Dates

1957			1958		
Treatment date	Herbicide	Dosage, kg/ha	Treatment date	Herbicide	Dosage, kg/ha
23 May	2.4-D	1.5	25 May	Tractor kerosene	780.0
14 June	2M-4Kh	2.0	23 June	2.4-D	2.0
10 July	Tractor kerosene	400.0	18 July	2.4-D	2.0
—	—	—	11 Sept.	2.4-D	2.0

Seven treatments with herbicides were made during two years in the rows of the belt (Table 1). The dosages of preparations 2.4-D and 2M-4Kh are indicated in conversion to the agent. The herbicides 2.4-D and 2M-4Kh were used as an aqueous solution, tractor kerosene was used undiluted. The control rows were weeded by hand. Weeding was done on the same days as the herbicide treatments.

The herbicides in the seedling rows were sprayed on the soil surface so that they did not fall on the sensitive parts of the woody plants, i.e., the points of growth, young shoots and leaves. In 1957 the growth of the oak seedlings was the same, both in the control and in the experiment. The treatment in 1958 with a considerably increased dose of tractor kerosene had a harmful effect on the oak seedlings and at the start of the season decreased their growth. However by autumn, growth had been recovered to a considerable degree.

The soil samples for analysis were taken twice during the 1958 season, on 25 July and 20 September. Each time five average developed oak seedlings were dug out of the treated and control rows. The soil samples for analysis were taken in the rhizosphere of the oak seedlings at a depth of 0-20 cm. A. N. Petrova made the analysis.

The calculation of the bacteria and fungi was conducted by the method accepted in the soil microbiological laboratory of the Institute of Microbiology, Academy of Sciences, USSR. The nonsporiferous bacteria were studied on meat infusion agar, the actinomycetes on starch-ammoniacal agar, the calculation of fungi was on acidulous wort agar.

The results of the investigation are given in Table 2.

First of all it is necessary to note that the data of the first and second dates of the calculation agree well with each other. This testifies to the sufficient reliability of the experiment results. The number of nonsporiferous bacteria in the rhizosphere of the control oak seedlings and the oak seedlings treated with herbicides was approximately the same. The number of actinomycetes in the rhizosphere of the seedlings treated with herbicides was somewhat lower than in the control. In the composition of the studied fungi were representatives of the genera Aspergillus, Fusarium, Mucor, Trichoderma, Penicillium and Rhizopus. The study of the fungi showed that Trichoderma predominated in the rhizosphere of the control oak

Table 2. The Microflora of the Rhizosphere of Two-Year-Old Oak Seedlings at a Depth of 0-20 cm in the Control Rows and Rows Treated with Herbicides (in thousands per gram of soil)

Culture medium	Microorganisms	Dates sample taken			
		July 25		September 20	
		control	experiment	control	experiment
Meat infusion agar	Total number of bacteria	2455	2052	1800	1702
Starch-ammoni-acal agar	Total number of bacteria	5800	4200	4431	3062
	Actinomyces	1503	900	1302	802
Acidulous wort agar	Total number of fungi	12	25	11	20
	Trichoderma	+++	++	+++	+
	Penicillium	5	15	4	12
	Aspergillus	2	4	1	3
	Fusarium	2	3	2	2
	Rhizopus	+	0	+	+
	Mucor	0	+	+	0

Notes: + weak development, ++ mild, +++ strong.

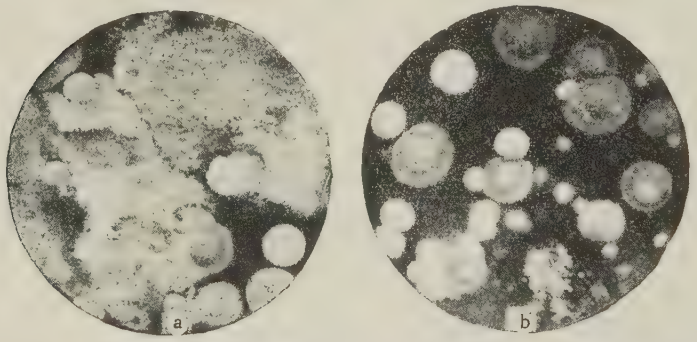

Figure. Fungi of the rhizosphere of oak seedlings. a) Control samples, Trichoderma predominates; b) samples treated with herbicides; Penicillium predominates.

seedlings. Penicillium and Aspergillus are considerably suppressed by this fungus. In soils treated with herbicides, Trichoderma is found in lesser numbers, it is poorly developed and its pigmentation is retarded. In this case representatives of the genus Penicillium are predominant and are developed somewhat better than in the control. A certain increase in the number of species of the genus Aspergillus is also noted. Trichoderma possess an antagonistic effect in relation to certain soil fungi (Kursanov, 1940), particularly to representatives of the Penicillium genus (Mishustin and Petrova, 1958). In the light of what has been said it becomes understandable that by suppressing Trichoderma in the rhizosphere of the oak seedlings which were treated with herbicides, the number of Pencillium and Aspergillus fungi increases (Figure).

Kursanov (1940) indicated that Trichoderma is the most active destroyer of cellulose and is characterized by an antagonistic effect on certain parasitic fungi which it crowds out, thus sanitizing the soil. In this book it is noted that Penicillium and Aspergillus also actively decompose cellulose in the soil and are able to crowd out other species of fungi including the parasitic ones. The noted changes in the soil microflora under the effect of 2.4-D apparently cannot have a substantial negative value. They did not have a noticeable effect on the condition of the woody plants. An effect by the tractor kerosene was not very likely since this herbicide did not penetrate deeply into the soil and quickly evaporated from it (Dekatov, 1958).

SUMMARY

Repeated treatments of herbicides: 2.4-D, tractor kerosene, and 2M-4Kh which were used for two years did not cause noticeable changes in the number of bacteria in the rhizosphere of the oak seedlings. A certain

decrease in the number of actinomycetes and a change in the specific and quantitative composition of fungi was noted.

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MICROBIOLOGICAL INVESTIGATIONS OF CARPATHIAN SULFUR DEPOSITS

II. STUDY OF THE MICROBIOLOGICAL PROCESS OF SULFATE REDUCTION IN THE ROZDOL SULFUR DEPOSIT

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The Rozdol sulfur deposit is the most thoroughly studied of the chain of Carpathian sulfur deposits (Kudrin, 1957; Sokolov, 1958), and during the period of our work was being intensively prepared for mining by the open-pit method. It was first of all necessary to remove hydrogen sulfide water from the sulfur-bearing stratum, for which water-reducing bores were drilled at the deposit and powerful pumps were installed (Fig. 1). Despite the fact that, by the summer of 1958, more than two static reserves of subterranean water had been pumped out of the deposit, the lower horizons of the sulfur-bearing stratum were still not freed of water. This fact indicates the intensive influx of water into the deposit, which apparently comes in from the west and northwest where the limestones do not have a clayey cover characteristic for the entire deposit, but come out directly under water-permeable Quaternary deposits.

The fact that, despite two washings of the sulfur-bearing stratum, the amount of hydrogen sulfide in the water being pumped out decreased relatively little (from 100–110 mg/liter in 1956 to 60–65 mg/liter in 1958) forced us to believe that the hydrogen sulfide reserves in the deposit are constantly being replenished due to its de novo production. Therefore, our problem at Rozdol included: 1) To investigate the distribution of sulfate-reducing bacteria in the deposit; 2) to determine whether conditions favorable for the vital activity of these bacteria exist in subterranean water, and 3) to determine the intensity of the microbiological process of sulfate reduction in subterranean water by means of labeled atoms.

A thick network of water-reducing and hydro-observation bores on the territory of the southern and central areas of the Rozdol deposit, as well as the presence of open workings made it possible to collect a mass of material, of which we were unfortunately deprived while examining Nemirov and Luben' (Ivanov, 1960), the exploration of which had already been completed, while their development had not yet begun.

The Distribution of Sulfate-Reducing Bacteria in the Sulfur-Bearing Rocks and Subterranean Waters of Rozdol.

Without stopping to describe the methods of the work which were set forth in the preceding communication (Ivanov, 1960), we shall go directly to the report of the factual material obtained. The data on the distribution of sulfate-reducing bacteria in the limestone of the Upper Tortonian horizon are presented in Table 1. We see that these bacteria were found almost along the entire cross section of the sulfur-bearing limestone both in bore No. 1 which is located in the center of the southern sector (Fig. 1) and which revealed a thick mass of sulfur-bearing limestone with argil

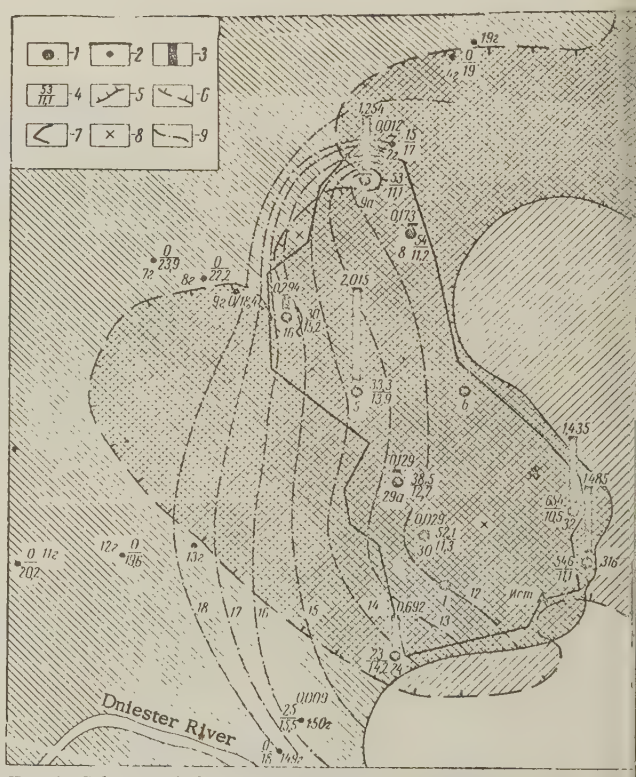

Fig. 1. Scheme of the central and southern sectors of the Rozdol sulfur deposit.
1) Water-reducing bores in which sulfate-reducing bacteria were found; 2) hydro-observation bores in which sulfate-reducing bacteria were found; 3) magnitude of the intensity of the sulfate-reduction process in mg/liter of H S per 24 hours; 4) numerator—H S content in mg/liter of water from bore, denominator—rH value of water; 5) contour of distribution of limestone water-bearing horizon; 6) contour of distribution of gypso-anhydrite stratum underlying the limestones; 7) border of industrial sulfur deposition in limestones; 8) places where cores were taken for the investigation of the distribution of sulfate-reducing bacteria; 9) rH isolines, drawn through the rH unit.

Table 1. Numbers of Sulfate-Reducing Bacteria in Core Samples from the Rozdol Sulfur Deposit

Sample No.	Bore No.	Brief description of core sample	Number of bacteria per 1 g
1	1	Dense nonsulfured limestone from upper part of limestone horizon	0
2	1	Cryptocrystalline sulfur from upper part of limestone horizon	0
3	1	Same as sample No. 2, but taken closer to the middle of the limestone horizon	1—10
4	1	Seams of dark carbonaceous organic material	10
5	1	from the middle of the limestone horizon	10
6	1	Sulfur from middle part of limestone horizon	10—100
7	1	Fragments of sulfured limestone in argillaceous seam in limestone	100
8	36	Fragments of slightly-sulfured limestone in clay	1000

Table 2. The Output and Chemical Composition of Water from the Bores Examined at the Rozdol Sulfur Deposit

Bore No.*	Output**	rH_2	O, mg/liter	H S, mg/liter	pH	Dry residue, mg/liter	SO, mg/liter	Date collected
4h	—	19.1	--	0	7.2	452	90	24.6
2h	—	17.0	0	17	7.0	1612	936	24.6
9a	2940	11.1	0	53	7.1	1802	1036	24.6
8	2445	11.2	0	54.5	7.1	1960	1111	24.6
7h	—	23.9	1.91	0	7.6	134	10	26.6
8h	—	22.2	0.54	0	7.4	234	57	26.6
9h	—	18.4	1.74	0	7.5	--	—	26.6
16	2000	15.2	0	30.6	7.0	1612	977	26.6
5	3920	13.9	0	33.3	6.9	2344	1297	26.6
19a	1370	12.7	0	38.5	6.8	2008	1332	2.7
30	0	11.3	0	52.2	7.1	1754	935	8.7
11h	—	20.2	2.04	0	7.7	—	—	12.7
12h	—	19.6	1.75	0	7.5	--	—	12.7
149h	—	18.0	1.76	0	7.4	184	41	2.7
150h	—	15.5	0	25	7.3	544	177	2.7
24	4220	14.2	0	23	7.2	2178	1378	2.7
Spring	—	11.0	0	50.8	7.3	2176	1342	5.7
31б	6100	11.1	0	54.6	7.2	2242	1312	5.7
32	2940	10.5	0	65.4	7.0	2252	1310	5.7

Note. *Hydro-observation bores are marked with the letter h following the number of the bore; **output indicated in m per 24 hours (average of last 10 days preceding the collection of the water sample).

laceous seams, and in bore No. 36, located on the western border of the outcropping of the ore body.

Sulfate-reducing bacteria were no less widely distributed in the subterranean waters of the limestone horizon. They were found in water from all of the bores without exception, both the water-reducing and the hydro-observation ones (Fig. 1). The most intensive and rapid blackening of the medium was observed when water from the bores of the southern sector (Nos. 24, 32, 31b, spring) and from the hydro-observation bores containing hydrogen sulfide water (150h, 2h, and 4h) was inoculated. It is especially important to emphasize, however, that sulfate-reducing bacteria were also found in those bores the water from which contained absolutely no hydrogen sulfide and even had dissolved oxygen (bores 7h, 8h, 9h, 11h, and 12h).

Thus, all these data show that sulfur-reducing bacteria are very widely distributed in the Rozdol sulfur deposit.

Conditions for the Vital Activity of Sulfate-Reducing Bacteria in the Subterranean Waters at Rozdol.

In order to form some idea of the possibility of the microbiological reduction of sulfates in the waters of the Rozdol sulfur deposit, we directed our attention to the study of the chemical composition of the subterranean waters at Rozdol. The results of this work are summarized in Table 2. We did most of the determinations (temperature and pH of the water, rH_2, and content of oxygen and hydrogen sulfide) right at the bore, immediately following the collection of the sample, and only the dry residue and sulfate content was determined at the laboratory of the Rozdol combine.

The data presented in Table 2 and selectively plotted in Fig. 1 show that, with respect to sulfate content and oxidation—reduction potential value, i.e., the two conditions which directly affect the activity of sulfate-reducing bacteria, all of the bores examined can be divided into two distinct groups. On the one hand, these are the hydro-observation bores Nos. 4, 7, 8, 9, 11, 12, and 149 which have a small content of sulfates, a high rH_2 (higher than 17), and do not contain hydrogen sulfide. All of these bores are situated beyond the contour of the gypsum which underlies sulfur-bearing limestones, and beyond the sulfur-bearing contours.

The second group includes all of the water-reducing bores and hydro-observation bores 150h and 2h which are distinguished by increased concentrations of sulfates, low rH_2 values, and a considerable content of hydrogen sulfide—from 15 to 65 mg/liter.

The data in Table 2 show that the salt composition and the oxidation–reduction conditions in the water from the bores of the second group are entirely favorable for the vital activity of sulfate-reducing bacteria. However, for the successful course of the microbiological reduction of sulfates, the presence of organic matter or oxygen is also necessary.

Different authors who have set forth theories concerning the origin of hydrogen sulfide in the Upper Tortonian waters of Carpathia have had different approaches to the question of the sources of organic matter necessary for the reduction of sulfates. Some of them have attributed the greatest significance to the organic substances of Quaternary deposits (Babinets and Rad'ko, 1950); others have proposed the migration of methane and other hydrocarbons from the direction of the depression (Sokolov, 1958); finally, still others have felt that the necessary organic materials are present in the sulfur-bearing limestones themselves (Kudrin, 1953; Gayun, 1956; Saidakovskii, 1955). However, the factual material on the amount of organic matter in sulfur-bearing rocks and on its suitability for microorganisms was extremely poor. Therefore, we decided to use the methodology of determining the biochemical oxygen demand (BOD) for the analysis of easily assimilable organic matter in subterranean waters. The results of these experiments are given in Fig. 2. We see that the BOD value is considerably higher in waters from the limestone horizon than in sandstone waters, and that this value increases as the waters move along the limestone horizon in the direction, bores 7h–8h–9h and 11h–12h. These data are evidence of the fact that the organic matter available for oxidation by microorganisms is washed out of the limestones themselves, rather than being brought into them from some place outside.

Thus, there are not only sulfate-reducing microorganisms in the waters of the Rozdol sulfur deposit, but all of the necessary conditions for their vital activity as well.

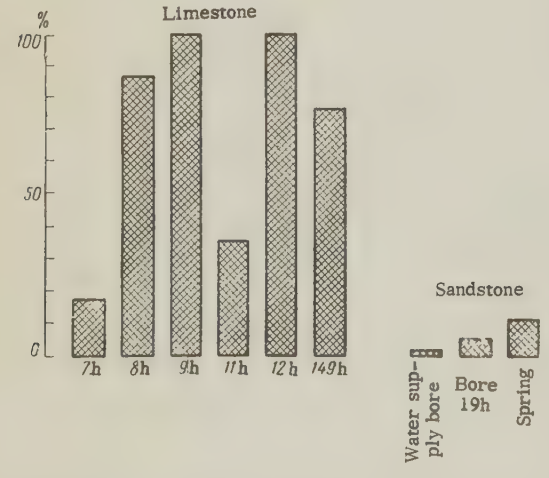

Fig. 2. Amount of oxygen consumed in per cent of its content at start of experiment. The water was artificially saturated with oxygen prior to the experiment. Interval—4 days.

The Intensity of the Microbiological Production of Hydrogen Sulfide from Sulfates in Subterranean Waters at Rozdol.

The concluding stage of our work was the determination of the intensity of the process of the microbiological reduction of sulfates, which was done by the same methods as at the Luben'-Velikii and Nemirov deposits (Ivanov, 1960). The results of these experiments are given in Table 3. Several conclusions can be made from the data obtained. First, the process of hydrogen sulfide production occurs only in the bores which we have related to the second group, i.e., in those where the rH_2 value is less than 17. This agrees well with the data which microbiologists have on the physiology of sulfate-reducing bacteria which are, as is well known, strict anaerobes. Second, the intensity of the microbiological process of hydrogen sulfide production from sulfates increases as the rH_2 value decreases (see bores 150h–24–31b, or bores 16–5 and 2h–9a).

At first glance, bores 8; 29a, and 30 serve as somewhat of an exception to this rule; the water from these bores has a very low oxidation–reduction potential, and at the same time, the intensity of hydrogen sulfide production in this water is very low. However, upon detailed examination, this peculiarity of these bores is easily explained. The fact is that bore 8 is located at the extreme eastern boundary of the central sector of the deposit (Fig. 1) and pumps water from the northern sector of the sulfur-bearing stratum which has not yet been touched by water-reducing pumping. Therefore, its water can not be compared with the water from the central and southern sectors of the deposit. Bores 29a and 30, however, although they pump water which is typical for these sectors of the deposit, are located in the difficultly washed and already nearly dried sectors of the deposit, which is indicated by the low output of these bores (Table 2). The encumbered influx of water to the face of the bore decreases the admission of organic matter brought by the waters into the stagnant subterranean basin which is formed and, consequently, worsens the conditions for the vital activity of sulfate-reducing bacteria in this basin. Apparently, the intensity of the process of hydrogen sulfide production from sulfates also decreases as a consequence of this.

Table 3. The Intensity of Hydrogen Sulfide Formation in Subterranean Waters at the Rozdol Sulfur Deposit (Summer 1958)

Bore No.	Duration of expt. hr	S/SO, mg/liter	Activity, thousands of cpm/min/liter		Intensity of H S production, mg/liter per 24 hrs
			S/SO_4	S/H_2S	
2h	34	312	4900	0,252	0,012
9a	35	345	6640	33,2	1,254
8	36	370	9970	6,6	0,173
8r	33	29	8100	0	0
9r	33	—	8300	0	0
16	34	326	8500	10,25	0,294
5	33	432	8050	49,0	2,015
29a	33	444	4850	1,80	0,126
30	36	312	7150	0,97	0,029
150r	35	39	5100	1,52	0,009
24	34	459	9450	19,00	0,692
31b	34	437	10600	48,00	1,485
32	35	437	9030	40,80	1,435

We should like to recall that a similar dependence of the intensity of the microbiological production of hydrogen sulfide on the output, and ultimately, on the rate of entry of fresh water to the face of the bore, was also noted by us at the Nemirov deposit (Ivanov, 1960).

In conclusion, it must be noted that the explanation suggested here for the connection between the intensity of the process of hydrogen sulfide production with the water-permeability of rocks and the output of the bore is still hypothetical.

SUMMARY

1. Sulfate-reducing bacteria are widely distributed in underground waters and in sulfur-containing rocks of the Rozdol sulfur bed. They are detected both in the hydrogen sulfide and non-hydrogen sulfide waters of the limestone water-bearing horizon.

2. A study of the chemical composition of waters and of the rate of the sulfate-reduction processes shows that sulfate-reducing bacteria produce hydrogen sulfide in underground waters only at a rH_2 value below 17.

3. The rate of sulfate reduction in underground waters of the Rozdol bed varies within a wide range, from 0.029 to 2.015 mg H_2S per 1 liter of water a day.

4. The diurnal intensity of microbiological reduction of sulfates depends on the oxidation-reduction potential of the water in question and apparently on the rate of water influx to the face of the bore.

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A STUDY OF THE HETEROTROPHIC PROPERTIES OF SPIRILLA GROWING IN THE FORM OF A MEMBRANE ON VAN NIEL'S MEDIUM

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Despite the fact that a great number of heterotrophic species of spirilla have been described, their physiology has been studied very little. In connection with this, it was of interest to determine the heterotrophic properties of a spirillum culture growing in the form of a membrane on van Niel's medium. As shown by preceding investigations (Vladimirova, 1958, 1960), the spirilla under investigation are capable of autotrophy (oxidize hydrogen sulfide to sulfates and utilize bicarbonate CO_2), and at the same time can grow on organic media without losing the capacity for subsequent change-over to the autotrophic mode of life.

The study of the heterotrophic properties of the given species became possible only after a pure culture of spirilla was obtained under conditions promoting the appearance of autotrophic properties, because when they were inoculated into organic media, intensive growth of contaminating heterotrophic forms occurred which prevented the growth of the spirilla.

A Study of the Growth of Spirilla on Various Organic Media.

When a pure culture of spirilla from membranes grown in test tubes of van Niel's medium was inoculated on plates of ordinary meat-peptone agar, colonies visible to the naked eye began to appear only after 10–12 days when the plates were kept at 25 deg and 30 deg. Regardless of the density of the inoculum, the colonies formed in this case were very small; they were dry, wrinkled, in the form of small mounds slightly raised above the agar and could be completely removed with a loop (Fig. 1). The same retardation in growth was also observed on MPB, where growth occurs on the bottom in the form of a small, thick, viscous sediment.

Inasmuch as the spirilla which were isolated from a pond are typical aquatic microorganisms, and the amounts of organic matter at their disposal are always considerably lower in natural conditions than on MPA or in MPB, it seemed expedient to study the growth of the spirilla on dilute organic media. Such cultivation on dilute organic media had been employed for the isolation of algal cultures (Goryunova, 1950) for example, as well as in Grey's work (1956), where several species of spirilla were isolated on solid medium with the addition of an extract of elm leaves, which in all probability can be regarded simply as the addition of a small quantity of some organic substances.

In the present work, wort diluted to 1 deg Balling and containing 1.5% agar was used as the medium with a low concentration of organic material. On such medium, colonies developed as early as the 2–3rd day. The colonies had the appearance of large, spreading, white or slightly pinkish, semitransparent tablets of somewhat indefinite form with slightly concentric accretion (Fig. 2).

In order to convince ourselves that good growth of the spirilla was observed specifically on dilute organic media, and did not depend on the quality of the organic substance, inoculations were made on dilute 1 deg Balling wort and on MPB and MPA diluted 10 times. Growth in the form of intensive and uniform clouding of the entire liquid was observed as early as the 2–3rd day after inoculation on both the wort diluted to 1 deg Balling and on MPB diluted 10 times. Colony growth was also noted as early as the 2–3rd day on MPA diluted 10 times. The colonies had the appearance of convex, completely transparent hemispheres (Fig. 3).

Thus, spirilla can grow as ordinary heterotrophic organisms, but in this case require media with lower concentrations of organic matter.

Since growth of the spirilla on ordinary MPA was very slow, and in enrichment cultures was inhibited by the growth of accompanying microorganisms, it was of interest to obtain a pure culture of spirilla on dilute organic medium. However, when a contaminated culture was plated on 1 deg Balling wort agar, after 2–3 days, only the growth of the contaminant forms was noted on the plates; nevertheless, colonies of spirilla began to appear several days later, sometimes directly under the colonies of the accompanying organisms (Fig. 4). By means of the selection of dilutions and by mass inoculation on plates, a pure culture of spirilla was obtained repeatedly which, as shown by tests, proved to be capable of the autotrophic form of life.

Aside from dilute MPB and wort, good growth of spirilla was found on medium containing peptone and glucose, where the mineral salts included in the composition of van Niel's medium served as the basic background (g/liter): K_2HPO_4—0.5, $MgCl_2$—0.2, NaCl—0.2.

When growing on such medium (as well as on other organic media), the cell morphology of the spirilla changed noticeably. Spirilla growing under autotrophic conditions on van Niel's mineral medium were large

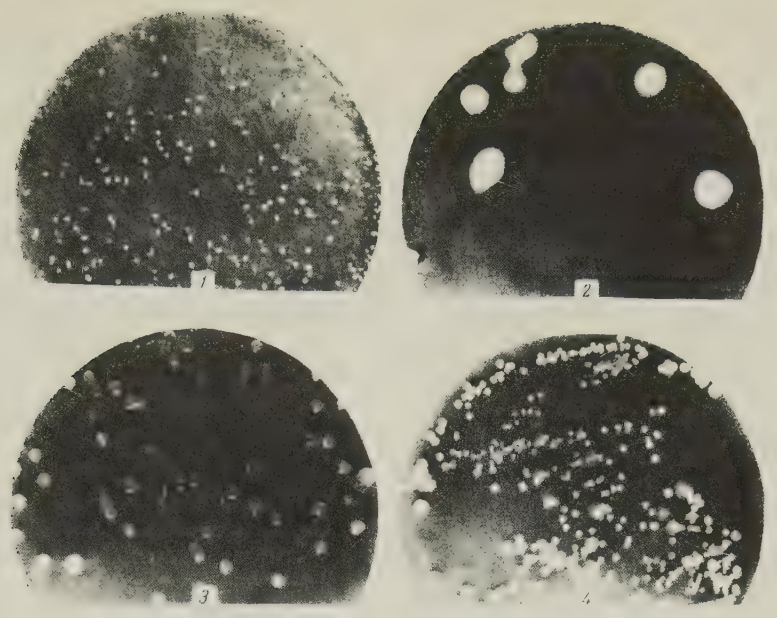

Fig. 1. Spirillum colonies on MPA (in 12 days after inoculation).
Fig. 2. Spirillum colonies on wort (1 deg Balling) in four days after inoculation.
Fig. 3. Spirillum colonies on MPA prepared with MPB diluted 10 times. In four days after inoculation.
Fig. 4. Colonies of spirilla and accompanying forms in six days after inoculation (on 1 deg Balling wort).

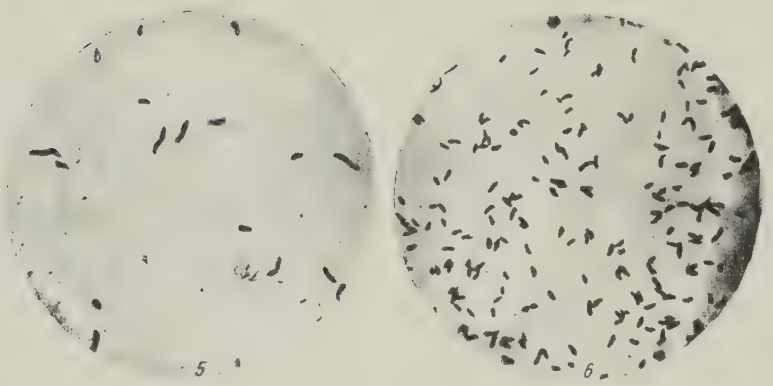

Fig. 5. Cells of spirilla from membrane growing in test tubes of van Niel's mineral agar medium flooded with water (6-day culture). Stained with erythrosine, fixed with alcohol.
Fig. 6. Cells of spirilla growing on liquid medium with 1% peptone and glucose (three-day culture). Fixed with alcohol, stained with erythrosine.

and predominantly S-shaped, while on organic medium, almost the entire culture consisted of small vibrios (Figs. 5 and 6). Spirilla from mineral medium stained with difficulty, while cells from organic media stained well with ordinary dyes.

Identification of Spirilla.

Inasmuch as we encountered no description in the literature of colorless spirilla capable of both heterotrophic and autotrophic ways of life, we attempted to identify the species under investigation only on the basis of its heterotrophic properties.

In addition to glucose, the spirilla grow well on maltose and xylose, and considerably poorer on lactose, galactose, rhamnose, and mannitol. Practically no growth is observed on sucrose. Acid production was found only on glucose and maltose.

When peptone was replaced by a mineral nitrogen source (NH_4Cl or KNO_3), growth in the presence of various sugars either became very much poorer or was absent.

In addition to growth on various sugars, the growth of the spirilla was studied on a number of organic acids in mineral medium in which peptone was replaced by 1% NH_4Cl. Good growth was observed only on lactic and malic acids. There was very poor growth on acetic, succinic, fumaric, oxalic, citric, and aspartic acids.

Thus, mineral sources of nitrogen are apparently not very favorable for the growth of spirilla, and can

serve as sources of nitrogenous nutrients only in the presence of certain carbon sources (for example, lactic and malic acids).

The spirilla grow well on a mixture of amino acids (very good growth on 1% casein hydrolyzate), as well as on dextrin.

The spirilla do not liquefy gelatin. Colonies on gelatin have the appearance of fine droplets or granules, sometimes growing inward slightly. Milk is coagulated to a slight extent and very slowly. Indole is not produced. Nitrates are reduced with the formation of nitrites and gases. The Gram stain is negative. The temperature optimum for growth on organic media is higher than for the autotrophic form of life. Thus, the growth optimum for the membrane in test tubes of van Niel's medium lies in the range of 20–25 deg, while on organic media, it shifts to 30 deg. Good growth is observed even at 37 deg.

In identifying the species both according to Krasil'nikov's key (1949), and according to Bergey's key (1948), the spirilla under investigation most closely approximate the species, Spirillum tenue Ehrenberg.

Growth of Spirilla on Liquid Medium Containing Peptone and Glucose.

Aside from the qualitative characterization of the heterotrophic properties of the spirilla, it is of interest to study their metabolism in greater detail. We conducted several experiments along these lines on medium containing peptone and glucose. As has already been mentioned, this medium is one of the best for the growth of the spirilla under investigation, and at the same time, is more convenient for analyses than such media as MPB, wort, or casein hydrolyzate.

First of all, a comparative study was undertaken of the growth of spirilla on such a medium as a function of the concentration of peptone and glucose.

The experiments were set up in 100 ml Erlenmeyer flasks. The media were dispensed in 50 ml amounts. After inoculation, the initial concentration of cells in the medium was 3–5 million per 100 ml. Cultivation was conducted at 27 deg for three days. When the experiment was terminated, the pH of the medium was determined potentiometrically, glucose—by Bierry's micromethod with preliminary precipitation of proteins with Barnstein's reagent, and volatile acids—by titration with 0.1 N NaOH after distillation with phosphoric acid. The growth of the culture was evaluated nephelometrically (in optical density units). When growing in liquid organic media, the spirilla formed substantial clumps which could not be broken up either by prolonged shaking or by the addition of acid; therefore, counts done on preparations made according to Vinogradskii led to very large errors, while nephelometric measurements gave results which were entirely comparable among themselves. For purposes of orientation, we shall nevertheless present several figures which give some idea of the relationship of the number of bacterial cells to nephelometer readings:

(a) Nephelometer readings	0.05	0.32	0.47
(b) Number of bacteria (according to Vinogradskii)	$0.5 \cdot 10^9$	$175 \cdot 10^9$	$640 \cdot 10^9$

The experimental results are given in Table 1, from which it is seen that when the spirilla grew on peptone alone, the increase in the number of bacteria was about the same on both the medium with 0.5% and with 1% peptone. In the presence of glucose, the addition of 1% peptone gave considerable further stimulation of the increase in number of bacteria. An increase in glucose concentration from 0.25 to 0.5% did not have much effect.

The same pattern, but with less intensity of the process (in connection with the decrease in peptone concentration) was observed in medium containing 0.1% and 0.5% peptone. It should be noted, however, that the production of volatile acids is in reverse relationship to the concentration of peptone.

It was determined by column chromatography that the volatile acid distillate contained only acetic acid.

A further increase in the concentration of glucose or peptone could have no further substantial effect either on the general course of the process or on the

Table 1. Growth of Spirilla on Medium Containing K HPO , MgCl , and NaCl as a Function of the Concentration of Peptone and Glucose

Composition of medium		pH		Glucose, mg/100 ml			Volatile acids, ml 0.1 N NaOH/100 ml			Growth of bacteria in optical density units on nephelometer
peptone, %	glucose, %	at start expt.	at end expt.	at start expt.	at end expt.	consumed	at start expt.	at end expt.	produced	
0,1	0	7,87	8,06	—	—	—	—	—	—	0,030
	0,1	8,56	8,23	16	Traces	16	—	—	—	0,13
	0,25	7,80	6,74	191	30	161	1,77	3,10	1,33	0,32
	0,5	8,37	6,97	374	253	121	2,21	6,19	3,98	0,37
0,5	0	7,35	8,20	—	—	—	—	—	—	0,10
	0,1	7,38	7,30	Traces	—	—	—	—	—	0,18
	0,25	7,33	5,91	200	8,3	191,7	1,59	2,21	0,62	0,35
	0,5	7,26	5,41	432	224	208	0,88	3,10	2,22	0,470
1,0	0	7,23	8,18	—	—	—	—	—	—	0,12
	0,1	7,18	7,97	Traces	—	—	—	—	—	0,35
	0,25	7,28	6,08	190	21,6	168,4	1,59	2,66	1,07	0,525
	0,5	7,06	5,48	419	205	214	1,77	3,71	1,94	Higher than 0.52 (about 0.62)

growth rate of the bacteria. Now, the question of the effect of aeration on the growth of the spirilla and the character of the alteration in the composition of the medium brought about by them was of considerably greater interest. In order to clarify this question, an experiment was set up with three variants with equal amounts of medium (75 ml) and inoculum, but with different degrees of aeration: in Vinogradskii flasks (height of liquid layer 0.7–1 cm), in 100 ml Erlenmeyer flasks (2.5–3.0 cm), and in narrow test tubes (15–20 cm); paraffin was poured into the latter and they were sealed with rubber stoppers. The results of the experiment are given in Table 2.

Table 2. Growth of Spirilla on Peptone with Glucose, as a Function of the Degree of Aeration*

Height of layer, cm	Nephelometer readings	pH	Volatile acids, ml 0.1 N NaOH per 100 ml	Glucose in mg/100 ml
0.7—1	0.521	6.56	6.46	18.25
2.5—3.0	0.279	6.71	8.32	31.3
15—20	0.006	7.3	1.91	284.3

*The initial pH value of the medium was 7.54, the glucose content 292 and the volatile acids 1.27 mg/100. The number of cells in the medium after inoculation was 5.5 · 106 per 1 ml (0.003 by the nephelometer).

It is seen from the experiment presented that the best bacterial growth (by increase in number of cells and glucose consumption) occurred in the most aerated conditions. Under relatively aerobic conditions, the same glucose consumption took place, but only half as much biomass accumulated in this case as compared with aerobic conditions. At the same time, the amount of volatile acids was less under aerobic conditions than in the variant with relative anaerobicity. In all probability, aeration promotes the more complete utilization of the oxidation products of glucose. Under anaerobic conditions, the spirilla did not grow at all. It was shown by column chromatography * of the volatile acids that only acetic acid was produced in all variants of the experiment, but in different quantities.

Thus, the spirilla grow well on medium containing 1% peptone and 0.5% glucose, are aerobes, and carry out the oxidation of glucose with the production of acetic acid.

In conclusion, I should like to express deep appreciation to Prof. V. N. Shaposhnikov and Prof. I. L. Rabotnova for constant attention and consultations during the performance of the present work.

SUMMARY

1. The heterotrophic properties of spirilla capable of the autotrophic form of life were studied; they were isolated from a bacterial membrane growing in test tubes of van Niel's agar medium covered with water.

2. The spirilla under investigation grow well as ordinary heterotrophs on media with a reduced concentration of organic matter.

3. The spirilla can utilize peptone, NH_4Cl, and KNO_3 as nitrogen sources; however, growth on mineral nitrogen sources is considerably poorer and depends on the carbon source.

4. The spirilla are aerobic organisms, and during growth on medium containing peptone and glucose, consume sugar with the production of acetic acid.

5. On the basis of the heterotrophic properties studied, the spirilla can be related to the species Spirillum tenue Ehrenberg.

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DISTRIBUTION OF MICROORGANISMS IN A BIOFILTER WHICH PURIFIES THE SEWAGE WATERS OF A HYDROLYSIS FACTORY

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The information available in the literature concerning the microflora of biofilters hardly mentions the processes of purification of industrial sewage, but rather pertains principally to the purification of farm and household water (Kalabina, 1934; Feldman, 1955; Hesseltine, 1953).

Occasional works deal with questions of the distribution of the film in the biofilter.

We studied the microflora of the biofilter during the purification of sewage waters from a hydrolysis factory. The purpose of the present work was to study the distribution of the microflora of the biological film in the biofilter both under conditions of its normal, uninterrupted work as well as after stoppage. The question of the effect of stoppage on the microflora of the biofilter was raised in connection with the fact that the interruptions occurring in the work of the factory frequently lead to the cessation of the supply of sewage to the biofilter.

Such stoppages sometimes last as long as 6-7 days. In the first days following the stoppage, purification proceeds poorly: contamination as determined by the BOD_5 decreases by 20%. Only after 10-15 days of uninterrupted work do the purification indicators become satisfactory and reduction in contamination reaches 80-90%.

Sugars and organic acids are the main impurities of hydrolysis sewage. Among the sugars, pentoses (xylose and arabinose) are represented in the largest amount, and hexoses appear in very small amounts. Of the organic acids, acetic acid is present chiefly, while formic and levulinic acids occur in very insignificant amounts. The organic acids are responsible for the acid reaction of the sewage waters. Waters prepared for purification has a BOD_5 of 400-500 mg/O_2, pH 5.8-6.5, a temperature of 21-26 deg, and contains 8-16 mg/liter of nitrogen and up to 2.5 mg/liter of phosphorus.

METHODS

Samples of crushed rock with the biological film were collected by means of digging to a depth of 5-15 cm and 40-50 cm along the height of the biofilter. The film was removed from the pieces of fill material with dissecting needles and was washed off with a known volume of sterile water. Dilutions were prepared from the suspension obtained after 10 minutes of shaking, and inoculations were made on nutrient media.

The greatest attention was devoted to the counting of microorganisms which oxidize sugars and organic acids. In addition to this, the number of microorganisms utilizing organic nitrogen was determined.

Solid synthetic medium of the following composition (in g) was used as the basal medium: agar-20, K_2HPO_4-1, $(NH_4)_2HPO_4$-1, $MgSO_4$-0.5, $CaCl_2$-0.1; NaCl-traces, $CuSO_4$-traces, tap water-1 liter. For counting the enumerated groups of microorganisms, xylose (1 g/liter), or acetic acid (0.5 g/liter), or peptone (5 g/liter), respectively, were added to this medium.

The inoculated media were kept at 22-24 deg for 3-6 days. When counting the number of microorganisms, the volume of the fill material from which the film had been washed off was taken into account. The number of microorganisms was calculated for 1 m^3 of crushed rock.

RESULTS

First, analyses of the microflora were made during the period of normal uninterrupted work of the biofilters, accompanied by satisfactory indicators of purification of the sewage water.

The results of the analyses revealed the following distribution of the microflora.

By total number of microorganisms, the upper layer was twice as rich as the deep layer (Fig. 1). This was also confirmed by data on the distribution of the biological film in the body of the biofilter (Table 1).

In the surface layer, molds, which constituted 53.5% of the total number of microorganisms, were the most richly represented. A large part of the molds was capable of oxidizing organic acids, while a smaller part belonged to the xylose oxidizers.

The second most numerous among the microorganisms of the surface layer were the bacteria, among which oxidizers of organic acids were found in large amounts, while bacteria oxidizing xylose and utilizing organic nitrogen occurred in smaller numbers. Yeasts were represented in insignificant amounts.

A different distribution of the microflora was observed in the deeper layer of the biofilter (40-50 cm). Of greatest importance in terms of numbers were the bacteria, 43.5% of the total number of microorganisms

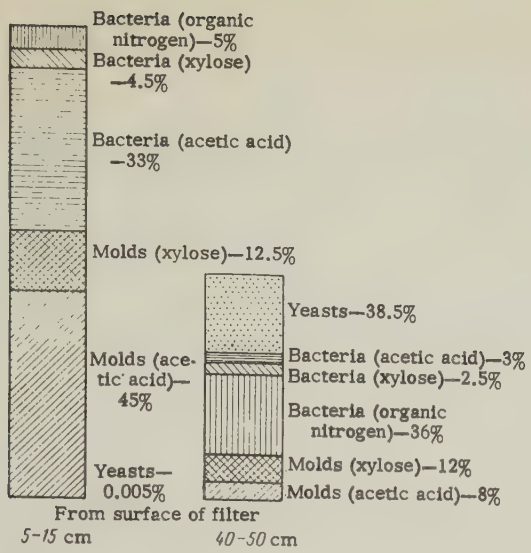

Fig. 1. Distribution of microflora in the body of the biofilter.

of this layer, and yeasts, constituting 39% of the entire microflora. The main part of the bacterial population is capable of living at the expense of the assimilation of organic nitrogen compounds, while bacteria oxidizing carbohydrates, xylose and organic acids, were represented in considerably smaller numbers.

The number of molds in the depth of the biofilter was considerably less than on the surface. Here, they constituted 20% of the total number of microorganisms.

Thus, the process of oxidation of xylose and organic acids goes on intensively in the surface layer of the biofilter. The total number of microorganisms carrying out these processes constituted 93.5% of the entire microflora in the surface layer, but decreased to 25.5% in the deep layer.

The reverse pattern was evident with regard to the bacteria which utilize organic nitrogen: there were 6% of them in the surface layer, while in the deep layer, their amount increased to 38%.

Table 1. Distribution of the Biological Film in the Body of the Biofilter

Biological film	Depth from surface of biofilter, cm	
	10–20	40–50
Amount of film in kg/m of crushed rock	25.4	9.8

Table 2. Content of Carbohydrates and Nitrogen in Sewage During Its Purification on the Biofilter

Sewage water	Xylose, mg/liter	Volatile acids in mg/liter of acetic acids	Nitrogen, mg/liter	
			organic	mineral —NH
Entering for purification	240	97.5	0	14.0
Having passed 15 cm	80	91.5	2.8	8.4
" 45 cm	30	29.4	11.4	1.4
" 75 cm	Traces	—	16.8	0
Purified	0	26.4	5.6	0

This was confirmed by the data on the change in the content of xylose, organic acids, and nitrogen in the sewage as it passed through the biofilter (Table 2).

During the time that the sewage water passes through the upper 45 cm layer, the major part of the carbohydrates has time to be oxidized, and further on, their amount decreases very slightly.

The incoming water contains only ammonia nitrogen which is rapidly used up by the biological film. At a depth of 45 cm, the ammonia nitrogen is consumed and organic nitrogen appears which is washed out of the biological film. A large amount of bacteria which assimilate organic nitrogen are concentrated here. As the result of their activity the amount of organic nitrogen decreases upon further flow of the sewage through the biofilter, and there is three times less of it in the purified water than in the water which has gone through 75 cm of the height of the biofilter.

Next, the effect of stoppages of the purification station on the microflora of the biofilter was studied.

It was established by many observations under industrial conditions that the cessation of the supply of sewage to the biofilter for as long as 36 hours does not affect the microflora and the oxidative power of the biofilter. Therefore, the effect of a longer stoppage was studied in the present work.

Analyses of the microflora were conducted after certain intervals of time following a seven-day stoppage of the biofilters. Four analyses were done: after 1, 4, 17, and 30 days. The results are given in Figs. 2–7. The purification indicators as measured by the reduction in BOD_5 (in%) changed in the following manner during this period: by 20 after 1 day, by 42 after 4 days, by 80 after 17 days, by 82 after 30 days.

Microscopic examination of the film after the stoppage and the results of the quantitative counts of microorganisms showed that a considerable dying off of the microflora had occurred in the surface layer of the biofilter.

Molds were completely absent on the surface, and were found in small amounts in the depth of the biofilter. The number of xylose- and acetic acid-utilizing bacteria was insignificant in the surface layer, but they were better preserved in the deeper layers. The same pertained to yeasts as well. Organic nitrogen-assimilating bacteria were preserved better than other groups in the deep layer of the biofilter.

An analysis of the microflora after four days following the stoppage showed that the restoration of the microflora of the biological film had begun in the surface layer. In this period, all groups of microorganisms grew vigorously on the surface of the biofilter: molds, bacteria, and yeasts, but the number of microorganisms decreased in going deeper into the mass of the biofilter.

The same picture was characteristic only for the first days of work of the biofilter following stoppage. Later (analysis after 17 days following stoppage), a distribution of microorganisms similar to that observed during normal uninterrupted work of the biofilter occurred: the microflora of the surface layer was represented chiefly by molds and bacteria of both types (those oxidizing xylose and volatile acids). In

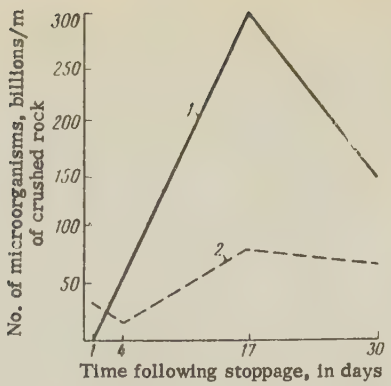

Fig. 2. Xylose-oxidizing molds. 1) Depth from surface of filter, 5-15 cm; 2) depth of filter, 40-50 cm. Conditional designations given for all figures.

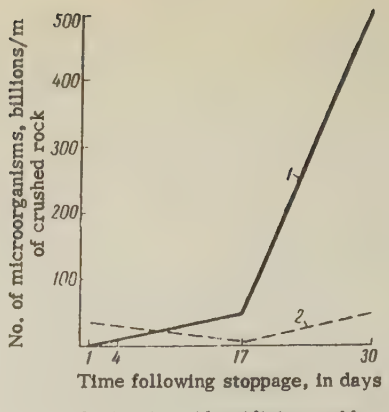

Fig. 3. Acetic acid-oxidizing molds.

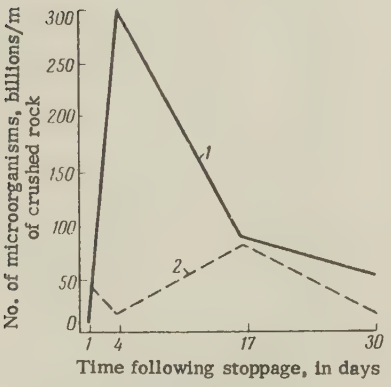

Fig. 4. Xylose-oxidizing bacteria.

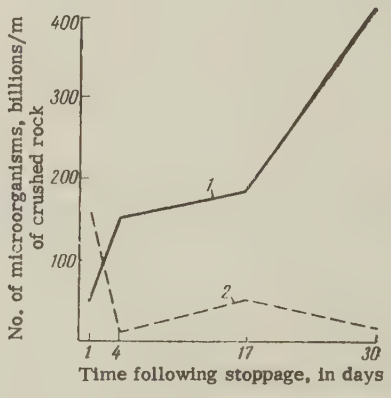

Fig. 5. Acetic acid-oxidizing bacteria.

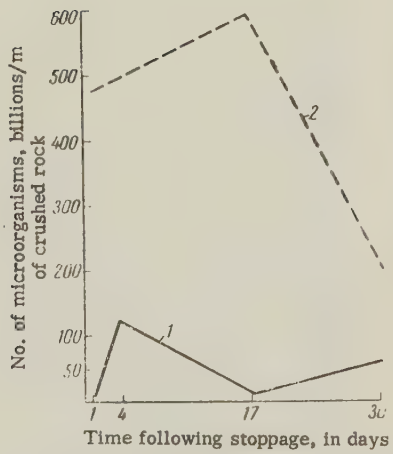

Fig. 6. Bacteria which utilize organic nitrogen compounds.

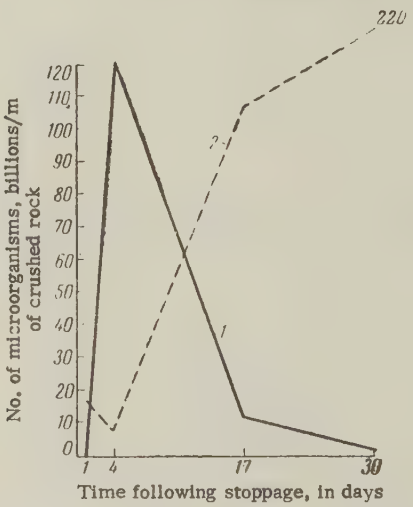

Fig. 7. Yeasts.

addition, some displacement of xylose-oxidizing bacteria by molds was observed in the surface layer in comparison with the preceding analysis. Putrefactive bacteria and yeasts were represented in considerable numbers in the depth of the biofilter.

After a month of uninterrupted work of the station, the normal distribution of the microflora in the mass of the biofilter was completely restored.

Thus, the following shift in the microflora was seen in the process of restoration of the biological film following stoppage. The molds which oxidize xylose and volatile acids (Figs. 2, 3) were practically absent in the surface layer following stoppage and were represented in insignificant numbers in the depth of the biofilter. During the very first days of normal work, the molds began to grow in the surface layer, and then their numbers increased greatly. In 17 days following stoppage, the molds were richly represented on the surface, while their numbers decreased in the depth of the biofilter.

With the resumption of work by the biofilters, xylose-oxidizing bacteria (Fig. 4) grew profusely on the surface; later, their numbers continued to increase in the depth of the biofilter as well. By the time that the microflora of the surface layer had been fully restored, these bacteria were partially displaced by intensively growing molds. The bacteria which oxidize acetic acid (Fig. 5) were retained in the depth of the biofilter following stoppage, and in considerably smaller amounts on the surface. Later, they continued to increase in numbers chiefly on the surface of the biofilter.

The bacteria which utilize organic nitrogen (Fig. 6) were concentrated in the depth of the biofilter. Their amount on the surface was not great following stoppage, and did not increase much later on.

Following stoppage, yeasts (Fig. 7) multiplied vigorously on the surface of the biofilter when it started its uninterrupted work; they were later displaced from the surface by molds.

SUMMARY

1. The distribution of the microorganisms of the biological film in the body of the biofilter was studied during the purification of sewage waters from a hydrolysis factory. The greatest number of microorganisms is concentrated in the surface layer (5–15 cm) of the biofilter. The amount of microflora decreases at a depth of 40–50 cm.

2. The microflora of the surface layer is chiefly represented by molds and bacteria which mineralize xylose and organic acids. With the exception of xylose-oxidizing bacteria, the number of these microorganisms decreases with depth.

3. Yeasts, xylose-oxidizing bacteria, and bacteria which utilize organic forms of nitrogen predominate in the depth of the biofilter.

4. A seven-day stoppage of the biofilter leads to the nearly complete dying out of the microflora of the surface layer, i.e., of molds and carbohydrate-oxidizing bacteria. In the depth of the biofilter, organic nitrogen-assimilating bacteria are well preserved, and a considerable number of yeasts and xylose-oxidizing bacteria remains. After stoppage, the indicators of purification become markedly worse.

5. During the first days after the resumption of work of the biofilter, all groups of microorganisms of the biological film multiply vigorously in the surface layer, and only after a certain 10–15-day period of work does the distribution of microorganisms characteristic for the normal working biofilter occur. By this time, the purification indicators become satisfactory.

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MICROBIOLOGICAL MACERATION OF EUCOMMIA LEAVES

III. DISINTEGRATION OF GUTTA AND RESINS IN THE PROCESS OF FERMENTATION OF THE LEAVES

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During the manufacture of gutta-percha from Eucommia leaves, the leaves undergo so-called "fermentation" or maceration (Rabotnova, Kupletskaya, and Kuznetsova, 1959, 1960). This is the microbiological stage of the manufacture during which the leaf tissues decompose, which facilitates the extraction of gutta-percha. A partial disintegration of the gutta-percha occurs at the same time. No information concerning the microbes which induce this disintegration is encountered in the literature.

The present work is devoted to the clarification of this question.

The gutta-percha extracted from Eucommia leaves consists of gutta and resins. The gutta is the needed product, while the resins are impurities which lower the quality of the gutta-percha. Gutta is a hydrocarbon, a derivative of isoprene C_5H_{10}. It is extracted by chloroform and is not soluble in acetone and alcohol. The substances which are extracted together with the gutta and are also soluble in chloroform, but can be separated from gutta due to their solubility in acetone and alcohol are called resins. The resins of gutta-percha-containing plants consist principally of unsaturated low-oxidation compounds, and have empirical formulas of the type: $C_{20}H_{44}O$, $C_{20}H_{30}O$, $C_{10}H_{16}O$. Regarding the resins of Eucommia leaves, it is only known that these are unsaturated compounds with very low acidity. During acetone extraction, colored substances from the leaves and lipoids also get into the resins (Voinovskii, 1951; Guseva, 1952).

METHODS

Microorganisms which disintegrate gutta and resins have been isolated from Eucommia leaves undergoing maceration. For this purpose, a selective medium was employed containing gutta or resins obtained from commercial gutta-percha as the sole carbon source and the following salts (in %): $(NH_4)_2HPO_4$–0.2, NaH_2PO_4–0.15, K_2HPO_4–0.1; $FeSO_4$, $ZnSO_4$, $MnCl_2$–traces, pH of the medium–7.0, tap water. For denitrifiers, 0.5% KNO_3 was added to this medium.

When agar medium was used, the resins or gutta were placed on the surface of sterile mineral agar in the form of a chloroform solution. The inoculation was made after the evaporation of the solvent. The resin and gutta were added to liquid media prior to sterilization. Autoclaving does not destroy resin and gutta.

For aerobic cultures, the liquid media were dispensed in a thin layer in 250 ml flasks, and pure asbestos was added in order to ensure uniform distribution of resins and gutta on the surface of the liquid. Otherwise, they stuck together in large clumps and sank to the bottom. For anaerobic microorganisms, the medium was poured up to the neck of flasks on the walls of which the resin had previously been spread. For greater anaerobicity, the flasks were sealed with Meissl traps filled with water.

The amount of gutta and resins was determined by the weight method after hot extraction with chloroform and acetone. In view of the fact that KNO_3 is slightly soluble in acetone, in the work with anaerobic cultures, gutta and resins were determined in material which was filtered off from the medium and washed to the disappearance of the reaction for nitrates. As shown by special tests, gutta and resins do not pass into the water.

RESULTS

The disintegration of gutta and resins goes on during the process of fermentation of Eucommia leaves, when the leaves are in a moist condition. If the leaves are kept in the dry form, the content of gutta and resins

Table 1. Changes in the Content of Gutta and Resins in the Process of Fermentation of Leaves (duration of experiments 1.5 months)

Eucommia leaves	Gutta		Resins	
	in mg per 5 g of original leaf	breakdown, %	in mg per 5 g of original leaf	breakdown, %
Original dry, not flooded with medium	116	—	258	—
Sterile in liquid medium	114	0	254	0
Fermented aerobically	97	15	141	45
Fermented anaerobically	114	0	221	14

Table 2. The Breakdown of Gutta and Resin by Fungi (duration of experiments 18 days)

Microorganisms	Gutta		Resin	
	in mg per 5 g of original leaf	breakdown, %	in mg per 5 g of original leaf	breakdown, %
Control (leaves before expt.)	135	—	243	—
Aspergillus strain 1	101	25	201	17
Aspergillus strain 2	105	22	207	15
Penicillium	89	34	208	14
Chetomium	101	25	215	11

does not change for a long period of time. Leaves moistened in the sterile state also do not lose gutta and resins. This is seen from the data in Table 1.

The results presented suggest that the breakdown of gutta and resins is not a simple physicochemical process of oxidation by oxygen from the air. This process is a microbiological one.

Breakdown of Gutta.

In order to detect the microorganisms which break down gutta, various samples of fermented Eucommia leaf were plated on mineral agar covered with a film of gutta. However, microorganisms did not grow. Then, small pieces of gutta were placed on the surface of the fermented leaves. After 2–3 weeks, they were overgrown by a film of microorganisms. From this, it was possible to isolate actinomycetes which further grew on gutta films. It was found that the film was completely destroyed under the actinomycete colonies.

In addition to this, when fermented leaves were plated on agar medium containing crushed leaf, a number of fungi were isolated which also break down gutta. They belonged to the genera Aspergillus, Penicillium, and Chetomium. When pure cultures of the fungi were inoculated on leaves moistened with a solution consisting of 0.2% $(NH_4)_2HPO_4$ and 0.1% K_2HPO_4, the fungi destroyed 22–34% of the gutta in 18 days (Table 2). They destroyed the resin simultaneously with the gutta.

Thus, the breakdown of gutta during the fermentation of Eucommia leaves under aerobic conditions is associated with the activity of fungi of the genera Aspergillus, Penicillium, and Chetomium. Actinomycetes can also utilize gutta.

The possibility of the breakdown of gutta during the fermentation of leaves under anaerobic conditions was checked. Fermentation was conducted with the addition of nitrates or sulfates, since denitrifying or sulfate-reducing bacteria may have been the gutta-destroying organisms. The leaves were flooded with a solution containing 0.2% $(NH_4)_2HPO_4$ and 0.1% K_2HPO_4 (background), or with this solution together with 0.5% KNO_3 or 0.5% $MgSO_4$. As seen from Table 3, gutta was not broken down under anaerobic conditions.

Breakdown of Resins.

The disappearance of resins from Eucommia leaves goes on not only during aerobic but during anaerobic fermentation of leaves as well. Therefore, a search was undertaken for aerobic and anaerobic resin-destroying organisms.

Aerobic Breakdown of Resins. Microorganisms capable of growing at the expense of resin were isolated from a number of samples of fermented leaf

Table 3. The Gutta Content of Leaves During Anaerobic Fermentation (duration of experiments 1.5 months)

Experimental variants	Medium	Gutta	
		in mg per 5 g of original leaf	breakdown, %
Original leaves	—	102	—
Leaves after anaerobic fermentation	Background	106	0
	" + KNO_3	106	0
	" + $MgSO_4$	110	0

Table 4. The Utilization of Resin by Aerobic Microorganisms During Growth on Resin as the Sole Carbon Source

Cultures	Resin in mg/flask		Resin broken down, %
	initial amount	after 3 weeks	
Aspergillus niger шт. 1	142	107	24
" niger шт. 2	141	113	20
" fumigatus strain 1	139	108	22
" fumigatus strain 2	140	110	21
" versicolor	142	104	26
" nidulans	142	106	25
Penicillium umbonatum	141	125	11
Trichoderma	142	123	13
Actinomyces iverini	142	113	20
" violaconiger	141	113	20
Mycobacterium lacticolum	140	139	0
Pseudomonas fluorescens шт. 1	105	80	24
" fluorescens шт. 2	105	81	23

by means of plating on mineral agar with a film of resin. In such platings, various fungi grew predominantly, while actinomycetes and bacteria were very rare. The cultures grew well in liquid medium containing resin as the sole carbon source. The fungi and actinomycetes covered all of the resin on the surface of the liquid with a thick mycelium. Data on the quantitative breakdown of resins by the cultures isolated from the growth due to resin alone are given in Table 4.

As seen from Table 4, fungi of the genera Aspergillus, Penicillium, and Trichoderma, actinomycetes, and bacteria of the genus Pseudomonas destroyed 11–26% of the resin in 3 weeks. Fungi of the genus Aspergillus, actinomycetes, and Pseudomonas were more active than Penicillium umbonatum and Trichoderma. Mycobacteria did not grow on pure resin.

Hardly any of the microorganisms listed in Table 4 grew on medium with gutta as the sole carbon source. Only Actinomyces violaconiger and both strains of Aspergillus niger gave poor, hardly noticeable growth on gutta.

The utilization of gutta in medium where it was the sole carbon source may have been limited by the absence of essential supplementary nutrient substances. In order to determine the possibility of the breakdown of gutta and resin directly in Eucommia leaves, the microorganisms were inoculated on sterile crushed leaves moistened with a solution containing 0.2% $(NH_4)_2HPO_4$ and 0.1% K_2HPO_4. All of the cultures grew well on the leaves. In this case, most of them did not touch the gutta of the leaves, although they were kept on the leaves for three months (Table 5). A. violaconiger and one strain of A. niger utilized gutta; however, the breakdown of gutta was not great. Meanwhile, 8–12% of the resin was broken down by many of the cultures. It proved impossible to determine the utilization of resins by the fungus Trichoderma, because either from the mycelium or from leaves altered by the fungus, some substances other than resins were extracted by the acetone. Some microorganisms, such as P. umbonatum and one strain of Aspergillus fumigatus for example, did not touch the resin during growth on leaves although they could grow on resin alone. Other microorganisms broke down resin to a slight extent. This is explained by the fact that, aside from resins, there are more easily

Table 5. Changes in the Content of Gutta and Resins in Leaves During the Growth of Aerobic Microorganisms on Them (duration of experiments three months)

Microorganisms	Gutta		Resin	
	in mg/5 g of original leaf	breakdown, %	in mg/5 g of original leaf	breakdown, %
Control (sterile leaves)	102	—	204	—
Aspergillus niger var. 1	103	0	188	8
" niger var. 2	97	5	178	12
" fumigatus strain 1	112	0	187	8
" fumigatus strain 2	108	0	200	0
" versicolor	101	0	182	11
" nidulans	106	0	182	11
Penicillium umbonatum	105	0	206	0
Trichoderma	104	0	—	—
Actinomyces iverini	99	0	186	9
" violaconiger	96	6	183	10
Mycobacterium lacticolum	104	0	185	9

Table 6. Change in the Resin Content of Leaves During Their Anaerobic Fermentation (duration of experiments 1.5 months)

Eucommia leaves	Medium	Resin	
		in mg/5 g of original leaf	breakdown, %
Sterile	—	227	—
After anaerobic fermentation	Background	193	15
	" $+KNO_3$	183	19
	" $+MgSO_4$	210	7

assimilable substances in leaves which are consumed by microorganisms first.

Some microorganisms can utilize resin in the presence of other nutrients. Mycobacterium lacticolum is among these. This bacterium did not consume resin and did not grow on medium with resin as the sole carbon source. However, when inoculated on Eucommia leaves, where the mycobacterium grows well, it began to utilize resin.

Part of the microorganisms isolated vigorously broke down both resin and gutta simultaneously. Some of the fungi given in Table 2 are among these.

Anaerobic Breakdown of Resins. The resins of Eucommia leaves are broken down not only under aerobic, but under anaerobic conditions as well. Inasmuch as the resins are substances which are extremely poor in oxygen, it would be difficult to assume their utilization by means of fermentation. It could be supposed that the anaerobic breakdown of resins proceeds due to oxidation by oxygen from nitrates or sulfates in the course of denitrification and sulfate reduction. In such a case, the addition of nitrates and sulfates could speed up the decomposition of resins. Eucommia leaves themselves contain few nitrates. Therefore, anaerobic fermentation of Eucommia leaves was carried out with the addition of nitrates and sulfates. The leaves were flooded with the following three media under anaerobic conditions: 1) Medium consisting of 0.2% $(NH_4)_2HPO_4$ and 0.1% K_2HPO_4—background; 2) background + 0.5% KNO_3; 3) background + 0.5% $MgSO_4$. Fermented Eucommia leaves were used for inoculation. Typical data of such experiments are given in Table 6. The breakdown of resins occurred even without the addition of sulfates or nitrates. The addition of nitrates increased the breakdown of resins. The addition of sulfates affected the entire fermentation unfavorably—the maceration of leaves became slower and the breakdown of resins was reduced at the same time.

When fermented leaves were inoculated into tall test tubes with resin and sulfate-containing medium, slight turbidity of the medium appeared. Vibrios, which could have been sulfate-reducing bacteria according to morphology, were found in the test tubes. However, their development was extremely poor. Judging by the fact that the addition of sulfates did not stimulate the decomposition of resins in leaves, while growth on resin flooded with medium containing $MgSO_4$ was extremely slight, even if sulfate-reducing bacteria do take part in the anaerobic breakdown of resins, it is not very great.

When fermented leaves were inoculated into tall test tubes with resin and KNO_3-containing medium, on the contrary, the medium became highly turbid and considerable gas production was observed. The decomposition of the resins of Eucommia leaves under anaerobic conditions apparently proceeds chiefly due to the denitrification process.

In order to isolate the denitrifiers, material from the enrichment cultures in test tubes was plated on mineral agar covered with a layer of resin. It was possible to isolate denitrifying bacteria which were capable of growing anaerobically on medium containing KNO_3 and resin as the sole carbon source. The five cultures which showed the most intensive growth on resin were selected; each strain was taken from a different sample of fermented leaf. Three of these cultures proved to be the bacteria, Pseudomonas fluorescens, while two belonged to Pseudomonas denitrificans. During anaerobic growth on medium containing KNO_3 and resin as the sole carbon source, the bacteria decomposed 12–25% of the resin in three weeks (Table 7). During this time, the number of bacteria increased from 150–300 thousand to 30–100 million cells per 1 ml.

The denitrifiers isolated did not grow on pure gutta either under aerobic or anaerobic conditions. At the same time, they decomposed 12–20% of the resin in the leaves (Table 8).

The denitrifiers consumed resin not only under anaerobic conditions, but could also oxidize it aerobically in the absence of nitrates. On resin-containing medium without nitrates, the three stains of P. fluorescens decomposed about 20% of the resins in three weeks under aerobic conditions (Table 4).

When the denitrifiers were cultured on meat-peptone agar, some of them lost their ability to utilize resin. This happened with P. denitrificans. The behavior of all P. fluorescens strains with regard to resin remained unaltered during four months. Later, the cultures were maintained on medium consisting of crushed Eucommia leaves (3–5%), agar, and mineral salts. The same medium was used during the period of a year to maintain the aerobic resin decomposers, which did not lose their ability to consume the resin of Eucommia leaves by the end of the year.

Table 7. The Utilization of Resin by Denitrifiers During Growth under Anaerobic Conditions on Resin as the Sole Carbon Source

Microorganisms	Resin in mg per flask		Breakdown of resin,%	Final number of bacteria, millions/ml
	initial amt.	after 3 weeks		
Ps. fluorescens strain 1	148	110	25	35
,, ,, strain 2	139	109	21	103
,, ,, strain 3	136	107	21	69
Ps. denitrificans strain 1	218	189	13	—
,, strain 2	218	192	12	—

Table 8. The Content of Gutta and Resins in Leaves During the Growth of the Denitrifiers on Them under Anaerobic Conditions (duration of experiments three months)

Strains of Ps. fluorescens	Gutta		Resin	
	in mg per 5 g of initial leaf	breakdown,%	in mg per 5 g of initial leaf	breakdown,%
Control (sterile leaves)	103	—	199	—
Ps. fluorescens strain 1	100	0	166	16
,, strain 2	102	0	156	22
,, strain 3	101	0	163	18

SUMMARY

1. It has been established that the breakdown of gutta and resins in the process of fermentation of Eucommia leaves is a microbiological process.

2. The breakdown of gutta is observed only under aerobic conditions and does not occur anaerobically. Aerobically, gutta is decomposed by actinomycetes and by molds of the genera Aspergillus, Penicillium, and Chetomium.

3. The breakdown of resins occurs both under aerobic and anaerobic conditions. Aerobically, the decomposition of resins is brought about by molds of the genera Aspergillus, Penicillium, Chetomium, Trichoderma, by certain actinomycetes, mycobacteria, and bacteria of the genus Pseudomonas.

4. The anaerobic breakdown of resins can be carried out by denitrifying bacteria of the genus Pseudomonas.

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A NEW VARIETY OF STREPTOCOCCUS LACTIS

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Streptococcus strain No. 18 which we isolated from corn silage (Sokolov, 1956) differs from ordinary lactic acid bacteria by its ability to decompose starch vigorously with the production of lactic acid (Fig. 1). The figure shows the growth of a seven-day culture of this strain on solid starch-containing nutrient medium with chalk; the culture had been treated with Lugol's iodine solution. In the figure, a zone of hydrolyzed starch can be seen which was produced by the action of amylase, and a zone where the chalk was dissolved by the lactic acid produced by the streptococcus. Orla-Jensen's works (1910) also contain information on starch-decomposing lactic acid bacteria. This circumstance prompted us to make a more detailed study of the morphological, cultural, physiological, and pathogenic properties of this microbe. The culture of this microbe is a streptococcus (Fig. 2), which sometimes forms long chains consisting of 25–30 or more cells. The cells are round and very small; the diameter of a single cell is about 0.7 microns, it does not have capsules, does not form spores, is not motile, and is Gram-positive.

The cultural characteristics were tested on MPB, MPA, and on MPG. Strain No. 18 forms uniform, hardly perceptible turbidity when growing in meat-peptone broth (the turbidity can be discerned only when the liquid is shaken). No gas or pellicle are produced in MPB. On MPA slants, it grows poorly forming a poorly-visible streak in the form of a slight whitish transparent film consisting of discreet, small, pin-point colonies. This film shows a pearly fluorescence in transmitted light. In MPB, the color and odor is not altered.

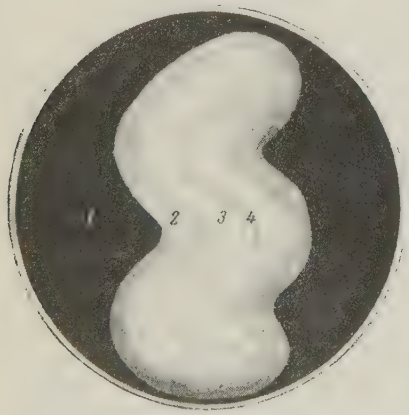

Fig. 1. Zigzag-shaped colony of Streptococcus lactis diastaticus (strain No. 18) on starch-containing nutrient medium. 1) Nutrient medium where starch has not been subjected to the action of streptococcal amylase; 2) zone of starch hydrolysis; 3) zones of chalk dissolution and starch hydrolysis; 4) zigzag-shaped streptococcus colony. Natural size (author's drawing).

Fig. 2. Streptococcus lactis diastaticus (strain No. 18) stained according to Gram. Magnification 1350x (author's drawing).

When a stab inoculation is made on MPA, the culture grows slightly along the entire length of the stab. On Petri dishes of MPA, it grows poorly in the form of very small pin-point colonies. The colonies are white, round, smooth, dense, with even, sharply demarcated edges. Somewhat larger slightly cloudy white colonies with diffuse edges are encountered among them. Pearly fluorescence is observed with transmitted light in both types of colonies. In all probability, strain No. 18 occurs in two forms: white and cloudy white; the number of white colonies observed was several times greater than that of cloudy colonies. Repeated transfers of both white and cloudy white colonies resulted in dissociation and the formation of the two kinds of colonies.

Gibshman (1948) and Nepomnyashchaya and Tevilevich (1955) pointed out the variability of lactic acid streptococci. When stab inoculations are made, the streptococcus does not liquefy gelatin and does not alter its color and odor.

An investigation of the behavior of this strain toward various carbohydrates showed that media with glucose, lactose, maltose, sucrose, raffinose, galactose, dex-

trin, and starch are fermented with acidification, but without gas production. Media with arabinose and xylose are fermented and acidified very slightly. Media with mannitol and glycerol are not fermented or acidified.

The microbe does not grow on synthetic medium with mineral nitrogen; it does not produce hyaluronidase or catalase. At 34–35 deg, milk is coagulated in 12–18 hours; the clot is compact, homogeneous, without gas production or peptonization. The acidity of the milk reaches 74 deg Terner on the third day after inoculation, and 90 deg Terner on the fifth day. Methylene blue is reduced. The streptococcus tolerates not more than 5% NaCl. It grows poorly on nutrient medium containing bile. The streptococcus is not destroyed when kept at 60 deg for 30 minutes. It ferments starch with the production of 1.05% lactic acid and 0.08% acetic acid; it does not produce butyric acid. It ferments glucose with the production of 1.18% lactic acid and 0.06% acetic acid; butyric acid is absent. It ferments lactose with the production of 0.98% lactic acid and 0.05% acetic; butyric acid is not produced.

It grows poorly on blood agar, forming a barely discernible streak; no zone of hemolysis is formed on the blood. When checked for pathogenicity (tests were conducted by A. K. Balitskaya on mice and young pigs), it was found that the streptococcus possesses no pathogenic properties. According to Krasil'nikov's (1949) and Bergey's (1936) keys, strain No. 18 should be related to the species S. lactis. The bacteria described by Orla-Jensen—S. amylolactis which decomposes starch with the production of gas and S. inulinaces which ferments mannitol with the production of acid—differ from the streptococcus isolated by us. It does not possess the indicated properties and belongs to a new variety of S. lactis which we propose to name S. lactis diastaticus.

SUMMARY

The streptococcus isolated from corn silage may be related to the Streptococcus lactis species.

In distinction from Streptococcus amylolactis described by Orla-Jensen which decomposes starch with the formation of gas, and Streptococcus inulinaces which ferments mannitol with the formation of acid, the above streptococcus is void of these characters and most probably belongs to a new variety which we propose to call Streptococcus lactis diastaticus.

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EFFECT OF THE VITAL PRODUCTS OF BACTERIA ON THE GROWTH AND ANTIBIOTIC PROPERTIES OF CERTAIN ACTINOMYCETES

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Many investigators have devoted much attention to the question of the production of antibiotic substances by actinomycetes.

It has been noted in a number of works that only 40-60% of actinomycete strains possess antibiotic activity, while the rest of the strains are inactive. Krasil'nikov's statement (1951) concerning the fact that every microorganism can apparently produce antimicrobial substances under appropriate conditions was confirmed experimentally with regard to actinomycetes by the works of Egorov (1956) and Makarovskaya (1956). While studying the conditions of growth and manifestations of particular properties in microorganisms, a number of authors (Izabolinskii and Soboleva, 1934; Krasil'nikov, 1951; Makarovskaya, 1956; Peretts and Slavskaya, 1933; Silishchenskaya, 1956; Streshinskii, 1949; Shen' Shan'-tszyun' and Shan' Vei-tszen, 1957; Dondero and Scotti, 1957; Grossbard, 1955) have investigated the effect of some microorganisms on the activation of growth or of particular aspects of the biochemical details of other microorganisms. It was shown that, during the joint cultivation of two or more microorganisms, an increase in the antibiotic activity of actinomycetes is sometimes observed (Makarovskaya, 1956; Shen' Shan'-tszyun' and Shan' Vei-tszen, 1957). However, the suggestions made to explain the increased biochemical activity of some microbes in the presence of others proved to be different. Some authors (Silishchenskaya, 1956; Shen' Shan'-tsyun' and Shan' Vei-tszen, 1957) believe that the main positive factor in the case under discussion is the direct effect of live cells of one organism on the other. In the opinion of these authors, the products of the vital activity of one of the organisms does not affect the activation of the biochemical activity of the other organism. Other authors (Makarovskaya, 1956; Dondero and Scotti, 1955) regard the stimulatory effect of one organism on another from the point of view of the effect of the products of vital activity of one of the organisms.

We adhere to the second point of view.

The purpose of the present work was to study the effects of the products of the vital activity of individual bacterial organisms on the growth and antibiotic activity of certain strains of mesophilic and thermophilic actinomycetes.

METHODS

A modified method of determining the antibiotic activity of microorganisms by means of a small agar block placed in the center of a Petri dish (Egorov, 1957) was used for the initial study of the effects of the products of vital activity of bacteria on the antibiotic activity of actinomycetes and on their growth; the method consisted of the following. The bacterial organism was inoculated on a meat-peptone agar block, while the actinomycete under investigation was plated as a solid background on the surrounding agar medium. A medium of the following composition was used in the experiments: glucose—10 g, KNO_3—7.2 g, $CaCO_3$—4 g, $MgSO_4$—2 g, K_2HPO_4—2 g, NaCl—5 g, tap water—1 liter, agar—20 g. After about 5, 8, and 12 days of cultivation, three agar blocks were cut out along the radii of the agar plate with a cork borer (6 mm diameter): the first close to the central agar block, the second in the intermediate space between the agar block and the edge of the Petri dish, and the third at the very edge of the Petri dish. Two variants were used as control: 1) The small agar block was not inoculated with the bacterium and, consequently, only the substances in the MPA could diffuse into the surrounding agar and cause the corresponding effects on the growth of the actinomycete; 2) the actinomycete was plated as a solid background over the entire surface of the agar medium which did not contain a MPA block.

In the experiments, only those species of bacteria were used which did not form antibiotic substances under our conditions. Staphylococcus aureus and Bacillus mycoides were used as test cultures in the determination of antibiotic activity.

EXPERIMENTAL

Study of Mesophilic Actinomycetes.

Bacteria and actinomycetes were isolated from a single sample of soil collected near Moscow. Certain interrelationships existing between them were established by the method described above. The results obtained here showed that the bacteria can exert different effects on the antibiotic activity of the actinomycetes. Some strains of bacteria increase it considerably or promote the production of an antibiotic substance in actinomycetes which did not produce it

under ordinary conditions of cultivation. Among the 27 strains of actinomycetes and 39 strains of bacteria used in our experiments, 11 cases of an increase in the existing activity or the new appearance of antibiotic activity in actinomycetes were noted. However, quite frequently no effect on the antibiotic properties of the actinomycetes was noted. In 18 cases, antibiotic production in actinomycetes either decreased considerably or disappeared entirely in the presence of bacterial organisms.

In many cases, the products of the vital activity of bacteria, without causing a noticeable change in the antibiotic activity of the actinomycetes, stimulated the growth of the mycelium, spore formation, or the production of pigments (23 cases). Sometimes, the growth of the actinomycete was inhibited, aerial mycelium disappeared, and spore formation was retarded (eight cases).

Two strains of actinomycetes were used for further work: actinomycete No. 31 which has a very low antibiotic activity and was identified (according to Gauze, 1957) as Actinomyces coelicolor, and Actinomyces sp., strain 87, which has a relatively high antibiotic activity. Of the bacterial organisms, the following were used: bacterium strain 22, related to Bacillus rusticus, strain 25, very similar to Bacillus liquefaciens, as well as Bacillus nitrificans and Achromobacter agile.

The further study of the effect of the products of vital activity of the bacteria mentioned on the antibiotic properties of the two strains of actinomycetes used was carried out according to the following scheme. The bacteria were grown for 1, 2, and 3 days on meat-peptone broth in 250 ml Erlenmeyer flasks. After this, the culture fluid from each culture was passed through a Seitz filter. One, 5, and 10% amounts (by volume) of the filtrate were placed in sterile liquid synthetic medium for growing the actinomycete; the medium was dispensed in 75 ml amounts in 250 ml Erlenmeyer flasks. The pH of the medium (7.0–7.2) was adjusted after the addition of the filtrate. The actinomycetes were cultured by the surface growth method at 28 deg. The antibiotic activity was determined by the dilution method. The following were used as controls: 1) Synthetic medium without any additions and 2) synthetic medium with 1, 5, and 10% MPB.

The experimental results obtained are given in Tables 1 and 2.

It is seen from Table 1 that the addition of culture fluid filtrate increased the antibiotic activity of A. coelicolor strain 31 3–9 times. One- and two-day filtrates were more effective as compared with three-day filtrates. A concentration of 1% proved to be the best filtrate concentration under the conditions of our experiments; as a rule, 5% filtrate was less effective, while the addition of 10% filtrate interfered with the biosynthesis of the antibiotic.

Table 1. The Effect of the Products of Vital Activity of Various Species of Bacteria on the Antibiotic Activity of A. coelicolor Strain 31, Which has Weak Antagonistic Properties (In dilution units)

Experimental variant	Time of cultivation of bacteria, days	Time of cultivation of actinomycete, days: 4				8				12			
		Amount of filtrate added, %: 0	1	5	10	0	1	5	10	0	1	5	10
MPB		0–6	18	18	0	0–6	18	0	0	0–6	6	0	0
Bac. rusticus	1		162	54	0		54	18	0		54	0	0
	2		162	18	0		54	18	0		54	0	0
	3		54	18	0		54	0	0		18	0	0
Bact. liquefaciens	1		54	18	0		18	0	0		0	0	0
	2		18	0	0		18	0	0		0	0	0
	3		18	0	0		0	0	0		0	0	0
Bact. nitrificans	1		162	54	0		54	54	0		54	18	0
	2		162	54	0		54	18	0		54	18	0
	3		54	18	0		54	18	0		18	18	0
Acromobacter agile	1		162	54	0		162	54	0		18	18	0
	2		162	162	0		54	54	0		18	18	0
	3		54	54	0		54	18	0		6	6	0

Table 2. The Effect of the Products of Vital Activity of Certain Bacteria on the Antibiotic Activity of the Actinomycete, Actinomyces sp. Strain 87, Which has Relatively High Antagonistic Properties (In dilution units)

Experimental variant	Time of cultivation of bacteria, days	Time of cultivation of actinomycete, days: 5				8				12			
		Amount of filtrate added, %: 0	1	2	3	0	1	2	3	0	1	2	3
MPB		54	18			54	54			18	18		
Bact. nitrificans	1		54	18	0		54	54	0		18	18	0
	2		54	54	0		54	18	0		16	6	0
	3		18	18	0		18	6	0		6	6	0
Achromobacter agile	1		162	54	0		54	18	0		18	18	0
	2		54	54	0		162	54	0		18	6	0
	3		18	18	0		18	6	0		6	6	0

The data presented in Table 2 show that the one-day filtrate of Achromobacter agile culture fluid stimulated the production of antibiotic substance by the actinomycete under investigation, especially during the first 5–8 days of its growth. B. nitrificans filtrate, on the other hand, had almost no stimulatory effect on the given actinomycete.

The stimulatory effect was more apparent in the low-activity actinomycete than in the relatively active one.

It was next determined which specific products of vital activity of the bacteria promote the increased production of antibiotic by the A. coelicolor culture. For this purpose, fractionation of one-day filtrates of the culture fluid from B. rusticus, B. nitrificans, and Achromobacter agile by the distillation method was undertaken. First, at a neutral reaction of the filtrate, the possible volatile neutral substances were driven off into distilled water. After this, volatile acids were distilled off (with steam) with the filtrate acidified with sulfuric acid. The residue from the distillation of the volatile acids was also used in the experiments after the sulfuric acid was precipitated with chalk.

The experiments were set up according to the following scheme.

1. Synthetic medium without additions (control).
2. Synthetic medium + 1% MPB (control).
3. Synthetic medium + 5% MPB (control).
4. Synthetic medium separate fractions from distillation and residue after distilling the filtrate of the culture fluid, added in amounts of 1 and 5%.
5. Synthetic medium mixture of volatile acid fraction and residue of distillation.

The results obtained are given in Table 3, from which it is seen that the stimulation of growth of the actinomycete, A. coelicolor, and the production of antibiotic by it due to the one-day filtrate of culture fluid from B. rusticus occurs as the result of the addition of the volatile acid fraction to the medium. Neutral products as well as the residue from the distillation of volatile acids inhibit the growth of the actinomycete under investigation and the production of antibiotic by it. Similar results were obtained when the fractions of one-day culture fluid from B. nitrificans and Achromobacter agile were studied with regard to their effect on the growth of A. coelicolor and the production of antibiotic by it.

The culture fluid filtrate from B. rusticus had a better stimulatory effect as compared with filtrates obtained when Achromobacter agile and B. nitrificans were cultured. Despite alkalization of the substrate to pH 8.0–8.4, B. rusticus produces a fixed amount of volatile acids (2.0–2.5 ml of 0.1 N NaOH per 10 ml of culture fluid) when cultured on MPB. An attempt to increase the amount of volatile acids by means of adding glucose to the MPB or by culturing the organism on synthetic media containing various sugars did not give positive results. It was possible to achieve a definite increase in the production of volatile acids by the B. rusticus culture (by about 2.5 times in comparison with the control) by shifting the initial pH of the medium to 8.2–8.6.

The culture fluid with the increased content of volatile acid was tested for the production of antibiotic by the actinomycete strain under study. The results obtained are given in Table 4.

The data given in Table 4 show that the increased content of volatile acids had practically the same effect on the production of antibiotics by the A. coelicolor culture as did their usual content (Table 3).

Study of Thermophilic Strains of Actinomycetes.

During the isolation from soil of thermophilic actinomycetes on the medium proposed by Kosmachev (1956) (KNO_3–1 g, $(NH_4)_2SO_4$–1 g, Na_2HPO_4–1 g;

Table 3. The Effect of Various Fractions of the Culture Fluid from B. rusticus Strain 22 on the Production of Antibiotic by an A. coelicolor Culture and on Its Growth (Antibiotic activity expressed in dilution units)

Experimental variant	Time of cultivation of actinomycete, days				Growth of actinomycete	
	5		8			
	Amount of added substance, %					
	1	5	1	5	1	5
Synthetic medium	0	0	0	0	Good	Good
Synthetic medium with MPB	18	0	18	0	Very good	Poor
One-day filtrate added	162	54	54	18	The same	Good
Volatile substances distilled at pH 7.0 added	0	0	0	0	Poor	Very poor
Salts of volatile acids added	162	162	54	162	Very Good	Very Good
Residue of distillation added	0	0	0	0	Poor	Poor
Salts of volatile acids+ residue of distillation added	54	18	18	0	Good	Good

Table 4. The Effect of Various Concentrations of Culture Fluid Filtrate from Bac. rusticus with an Increased Content of Volatile Acids on the Production of Antibiotic by an Act. coelicolor Culture (In dilution units)

Experimental variant	Time of cultivation of actinomycete, days											
	4						8					
	Amount of substance added, %											
	0	0,1	0,5	1	5	10	0	0,1	0,5	1	5	10
MPB	0	0	18	18	18	0	0	0	18	18	0	0
Filtrate		0	54	162	162	18	—	0	18	54	162	18
Volatile acid salts		0	18	54	162	54		0	54	162	162	18

Table 5. The Effect of Bacillus sp. and Its Culture Fluid on the Growth and Antibiotic Activity of a Thermophilic Actinomycete Strain

Number of experiments	Experimental variants	Time of cultivation of actinomycetes					
		3 days			10 days		
		Growth of actinomycete	Antibiotic activity on		Antibiotic activity on		Growth of actinomycete
			Staph. aureus	Bac. mycoides	Staph. aureus	Bac. mycoides	
3	Actinomycete	Poor	++	+	+	+	Poor
	Actinomycete and bacterium	Good	++++	+++	++++	+++	Good
2	Actinomycete	Poor	+	+	+	+	Poor
	Actinomycete and bacterial culture fluid	Good	+++	+++	+++	+++	Good

Note. + zone of growth inhibition very small; ++ zone 12 mm; +++ zone 15 mm; ++++ zone 20 mm.

$MgSO_4$—0.5 g, $FeSO_4$—0.001 g, $CaCO_3$—4 g, starch—20 g, tap water—1 liter, agar—20 g) and during their further purification, the following phenomenon is observed. The actinomycetes grow quite well around the soil clumps, but growth becomes considerably poorer away from the clumps. When the actinomycetes are transferred to fresh nutrient medium, they also show poor growth. The replacement of the starch-ammonia medium by other media did not give positive results with regard to improving the growth of the actinomycetes.

The addition of from 1 to 3% of an aqueous soil extract to the starch-ammonia medium improved the growth of the thermophilic actinomycetes considerably. However, the products of vital activity of certain soil bacteria had a better effect on the growth of the thermophilic actinomycetes and on the production of antibiotic substances by them. Thus, when a thermophilic strain of an actinomycete of the genus Micromonospora was accidentally contaminated by the spore-forming bacterium Bacillus sp., it was found that growth of the actinomycete improved considerably and its antibiotic activity increased.

Under our experimental conditions, the spore-forming bacterium isolated had no antibiotic activity with respect to the test-microbes which we used—S. aureus and B. mycoides.

As the result of the series of experiments conducted (Table 5), it was determined that not only the joint cultivation of the actinomycete with the isolated bacterium, but its culture fluid as well has a substantial effect on increasing the antibiotic activity of the thermophilic actinomycete strain and improves its growth considerably.

SUMMARY

1. The vital activity products of various bacterial species affect growth of both mesophilic and thermophilic strains of actinomycetes as well as the production by them of antibiotics. The vital activity products of some kinds of bacteria stimulate growth of actinomycetes and the formation of antibiotics while others inhibit both processes. The products of still other species do not exert any action whatever either upon growth of the actinomycetes or upon the production of antibiotics.

2. In the presence of the products of certain bacterial species, the stimulating effect upon the production of antibiotics is more pronounced in so-called inactive or slightly active actinomycetes strains and less pronounced or altogether absent in relatively active strains.

3. The stimulation of the production of antibiotics by mesophilic actinomycetes strains is linked with the volatile acids fraction obtained from one-day culture fluid of some bacterial species.

4. The addition to synthetic medium of a bacterial culture fluid with an increased content of volatile acids does not stimulate the production of antibiotics by the actinomycetes strain at issue as compared with a culture fluid containing the usual amount of volatile acids.

5. The addition to starch-ammonia medium of 1–3% aqueous soil extract stimulates growth and production of antibiotics by thermophilic strains of actinomycetes.

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CHANGES IN CHEMICAL AND MECHANICAL PROPERTIES OF PAPER WHEN IT IS AFFECTED BY THE FUNGUS GYMNOASCUS SETOSUS

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It is well known that microorganisms can damage paper during storage and can change its properties profoundly.

The destructive activity of fungi and bacteria in paper pulp, cotton, and cellulose products has been frequently pointed out in the literature. In paper pulp made of conifer wood, bacteria reduce the content of α- and β-cellulose noticeably in two weeks, leaving the amount of lignin unchanged (Ayer, Ford, and Smilie, 1957). The viscosity of cellulose solutions, on the contrary, is increased; the bacteria evidently first use the cellulose which has short-chained molecules. As the result of the chemical testing of jute bags, yarn, and fibers destroyed by mold, Macmillan and Basu (1947) came to the conclusion that, due to microbiological processes, the ability of jute fiber to dissolve in alkali increases about three-fold, the pH decreases from 6.5 to 5.5, while the absorption equivalent for methylene blue remains normal. The fungi utilize cellulose and hemicellulose, leaving lignin untouched. The damaged cellulose has an increased solubility in 7-11% NaOH, and the shorter the molecular chain, the more the solubility rises (Grethouse, 1950). A definite relationship exists between the solubility of altered cotton in alkali and the viscosity of its cuprammonium solution (Birtwell, Clibbens and Geake, 1928). Reducing sugars can not be found during the hydrolysis of cellulose; they are apparently immediately consumed by the fungi. The weight loss is usually relatively small, but the mechanical properties are considerably reduced, while the copper number increases (Siu and Reese, 1953).

Our work is an attempt to characterize as completely as possible the changes occurring in paper when the fungus Gymnoascus setosus is growing on it.

METHODS

The paper affected by the fungus was tested for resistance to breaking, puncture, and shredding, as well as for tensility and tearing length. Then, its ash content, α-cellulose, degree of polymerization of the cellulose, carboxyl groups, copper number, solubility in 1% NaOH at room temperature and with boiling were determined. The work was conducted according to the methods accepted by the Central Scientific Research Institute of the Paper Industry (Handbook for the Paper Technologist, 1955). In determining the degree of polymerization of cellulose, we followed the directions in the article by Lyubimova et al. (1957).

All analyses were done with 2-5 parallel samples and were repeated 2-3 times until completely convergent results were obtained.

EXPERIMENTAL

Filter paper prepared from wood, cotton, and flax cellulose was used as the test material. Disks of this paper in Petri dishes were moistened with nutrient medium containing (in %): sucrose-1, peptone-0.2, and ammonium molybdate-0.02. This medium promoted good pigment production by the fungus, and was taken as standard in the experiments. The paper to be used for mechanical tests was cut into strips and squares of the appropriate size. All samples were examined after two months from the day of inoculation, when they were strongly colored by the fungal pigments. They were then thoroughly washed with distilled water in order to remove residual nutrient medium and were dried under a press. The samples were subjected to mechanical tests in this form. The test paper underwent additional preparation for chemical analyses. First of all, it was cut into parts according to the degree of affectation by the fungus. For the investigation, those portions of the paper were selected which could be related to the following three groups: 1) Severely broken down paper which had turned brown, on which the central part of the fungal colony had been located; 2) paper intensively stained by the fungus, directly adjacent to the place where the central part of the colony had been; 3) completely unstained paper which had been beyond the limits of the colony.

The paper divided in this manner was mixed in water to the fibrous state and molds were prepared. All the molds of a single group were broken up into small pieces which were mixed together and used for the analyses as an average sample.

The condition of the fiber in each series of molds is shown in Figures 1, 2, and 3, in which the following can be seen: 1) In paper which was occupied by the central part of the fungal colony, small, short, severely damaged fibers predominate. Nearly all of them are pigmented. Long fibers are rarely encountered here, while unstained ones are nearly absent; 2) the paper of the next zone is less disrupted, but is stained strongly, consists of longer fibers part of which have not been affected by the fungus at all, and has retained its

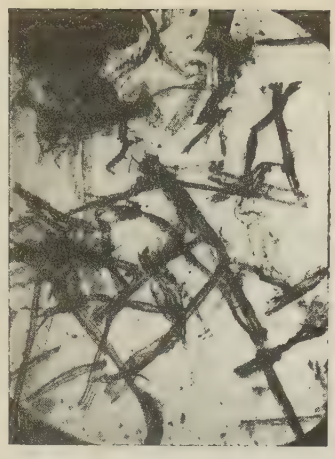

Fig. 1. Paper fiber in place where central part of the colony was located. Magnification 120×.

Fig. 2. Paper fiber in zone of strong pigmentation. Magnification 120×.

Fig. 3. Paper fiber beyond the limits of the colony. Magnification 120×.

structure. Along with these there are uniformly red fibers which are shortened and deformed as in the previous case, but they do not predominate; 3) the paper beyond the pigment spots consists of long fibers which have retained their structure and are devoid of stain. Single pigmented fibers can be found occasionally. Basically, the composition of the fiber in this paper is the same as in the original undamaged samples which serve as control.

Having distinguished three zones on the damaged paper, it was possible to do away with average indicators and to determine the changes in its properties in different portions depending on the location of the fungal colony. It should also be kept in mind that, during the two months of growth of the fungus, the paper lost an average of 19.4% of its initial weight. This loss was apparently also distributed unevenly by zones, and the greatest loss in weight was in the center of the colony where the paper was frequently completely broken down.

Practically, the second zone which bears the main pigmentation, contains much mycelium, and has insignificantly altered mechanical properties is the most meaningful; it is precisely this zone which has to be decolorized when removing spots from paper. Some amount of cellulose is completely broken down in this zone as well, but it is insignificant.

The results obtained from mechanical tests and chemical analyses are given in the table, each figure of which represents the average, most characteristic value of all parallel tests. In examining the data of each column of the table along the vertical, from the bottom up, the alteration in the properties of the paper can be followed from the periphery to the center of the colony, and the deviations of these samples as contrasted with the original paper can be determined. The samples were not divided into groups for the determination of mechanical properties. The data of these tests are a composite for the whole sample, although they do not always correspond to this. The tests for tearing length and resistance to puncture in which, due to the technique of the test, the break occurs in the weakest part, can be attributed to the most disrupted part. The situation is different with the determination of resistance to breaking and shredding, where the part of the paper belonging to any of the three groups, depending on the position of the colony on the test sample, can be tested. This was evidently reflected in the results as well: an especially sharp increase occurred in the determination of tearing length and resistance to puncture. Tests for breaking and shredding revealed considerably smaller changes in comparison with the control.

DISCUSSION OF RESULTS

First of all, the fact that changes occurred in the material designated as sample group III, i.e., in the paper participating in the experiment but outwardly having no traces of affectation by the fungus, deserves attention. The degree of polymerization of cellulose here decreased from 866 to 670; the solubility in alkali increased by 15–30%. Other indicators changed very little, while carbonyl groups (copper number) and the number of carboxyl groups remained the same as in

Table. Determination of the Chemical and Mechanical Properties of Paper Affected by Fungus

Zones [illegible] by the fungus	Moisture, %	Ash content, %	α-cellulose, %	Solubility in 1% NaOH at room temperature, %	Solubility in 1% NaOH with boiling, %	copper number	Degree of polymerization	Carboxyl groups, %	Resistance to breaking, number of double folds	Tearing length, m	Tearing load, kg	Tensility, %	Resistance to shredding, g	Resistance to puncture, kg/cm²	Loss of weight %
I	4.62	0.29	78,8	2,41	13,57	1,69	519	0,45							19.4
II	4.50	0.23	83.5	1.65	9.80	1.18	620	0,14	3	890	1, 2	0.83	45.5	0.018	
III	5,17	0,18	88.4	0.86	7,69	0,82	679	0,12							
Control	5,37	0,16	90.6	0.67	6,67	0.82	866	0.12	4	1711	2,03	1, 1	69.5	0.283	—

the control sample. Thus, sterilization and two months of being kept in a moist condition under the influence of nutrient medium and the metabolic products of the fungus were not without effect and reflected not on all, but on some of the chemical properties of the fibers.

In the material related to the second group which consists of strongly pigmented paper, the changes affected all of the indicators without exception. Solubility in alkali rose 1.5–2.5 times, the degree of polymerization of cellulose dropped to 620, the amount of -cellulose decreased by 7%, the copper number increased considerably, while the content of carboxyl groups constituted 0.14%. It is obvious that profound chemical changes are characteristic for this type of breakdown of paper. In this case, oxidation proceeded only to the aldehyde stage, while carboxyl groups increased insignificantly.

In the first group of samples which includes the most disrupted parts of the paper, which had disintegrated completely in places, these changes went even further. Solubility in alkali increased 2–3.5 times, the content of -cellulose decreased by 12%, while the degree of polymerization of cellulose decreased by 347 units. The rise in the per cent of carboxyl groups was especially sharp—more than 3.5 times—which makes the test sample approximate oxycellulose.

In comparing the analytical data, it should be noted that the differences among the three groups of fibers under investigation were particularly marked in the determination of solubility in alkali and degree of polymerization. Large deviations of all indicators were found in the samples which were severely broken down by the fungus, especially the percent of carboxyl groups. The increase in the functional groups, COH and COOH, in cellulose is evidence of its profound chemical change and in large measure occurs due to humified cellulose, which is insignificant in weight, but has a high acidity. The ash content evidently increased because of the accumulation of a large mass of mycelium contained in the samples of cellulose and the amount of which increases as the destruction process proceeds.

The samples being analyzed represent the part remaining after the complete breakdown of nearly 1/5 of the initial cellulose. In connection with the fact that the destruction of the paper does not occur diffusely, but rather focally, the extent of destruction of the individual fibers depends on the location of the colony and differs by zones within the limits of a single colony. In the zone where the mycelium occurs, the destruction affects all types of fibers, both small and large. The latter become considerably shortened and lose their morphological structure. In the middle portion of the colony, the paper fibers turn into a formless mass. The fungus evidently utilizes cellulose of different chemical compositions including α-cellulose and cellulose of a high degree of polymerization.

The results presented on the investigation of the three groups of affected paper can also be regarded as stages in the changes occurring in cellulose under the influence of the vital activity of the fungus. Taken together, they characterize those peculiarities which distinguish the process of the biological destruction of fiber in paper.

In conclusion, I should like to offer my gratitude to F. P. Komarov for consultations concerning the chemical methods of testing cellulose and to V. M. Efimova for assistance in carrying out the analyses.

SUMMARY

1. Decomposition of paper fibers by Gymnoascus setosus varies from hardly noticeable chemical and morphological changes to complete disintegration.

2. Morphological examination, mechanical tests, and chemical analyses are necessary for the characterization of the changes occurring in paper as the result of the activity of fungi. The chemical analyses make it possible to detect fine changes in the fiber, while mechanical tests are too crude for this and can give haphazard results.

3. Paper damaged by the fungus is distinguished by profound chemical changes which are characterized by a great increase in the percent of carboxyl groups, copper number, solubility of cellulose in alkali, and a decrease in the degree of its polymerization.

Moreover, the paper loses its mechanical properties to a considerable extent, especially resistance to puncture and tearing length.

4. The destruction involves not only minute but also large fibers which are appreciably shortened.

5. Within two months, the fungus destroys about 20% of the initial cellulose completely, using α-cellulose as well.

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EFFECT OF BACTERIA ON THE ELECTRODE POTENTIAL OF STAINLESS STEELS IN SEA WATER

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The number of saprophytic bacteria existing in sea water in the suspended state does not exceed 1–5 cells per 1 ml of water. When glass slides are submerged in sea water, bacteria settle on them and, multiplying rapidly, reach values expressed in thousands of cells per 1 cm^2 of surface in several days. A similar picture is also observed when steel plates are submerged in sea water. Therefore, the vital activity of bacteria growing directly on the passive film of stainless steels, which measures 10–50 A in thickness (Akimov, 1956) must to some extent affect their resistance to corrosion. The latter is determined chiefly by the surface passive film, the protective properties of which are to some extent characterized by electrode potential. The more positive the electrode potential, the greater the protective properties of the passive film, and the reverse (Tomashov, 1952).

When bacteria grow in discrete spots on the surface of stainless steel, the electrode potential of these areas shifts to the negative side. Aside from this, galvanic couples may arise which would lead to intensive local destruction of the steel.

The purpose of the present work was to study the effect of bacteria on the protective properties on the passive film of stainless steels.

METHODS

Three series of experiments were set up. In the first series, the water was inoculated with a culture of Vibrio desulfuricans, in the second, with Pseudomonas fluorescens liquefaciens, and in the third, with Leptothrix crassa. The experimental flasks were inoculated with bacteria by the following method. To the flasks of the first series, 10 ml of a 10-day culture of V. desulfuricans on Tauson's liquid medium was added. To the flasks of the second and third series, 2 ml of a suspension of P. fluorescens or L. crassa cultures obtained by washing off 2–3-day agar cultures with 10 ml of water was added. To count the bacteria which had grown on the steel sample, its surface was scraped into 5 ml of sterile water (Rozenberg, Ulanovskii, and Korovin, 1959). The number of sulfate-reducing and iron bacteria was determined by microscopic examination of smears stained with carbolic erythrosine, while P. fluorescens was counted by the plate method on MPA. To measure the electrode potentials irregular-shaped samples of steel 1Kh18N9T and 1Kh13 were placed in glass flasks containing 1 liter of sea water (see Table 1) and the equipment illustrated in Fig. 1 was assembled. The surface of the samples was polished and was treated with alcohol and flamed over an alcohol burner prior to the experiment. Water from the Black Sea served as the medium for the test.

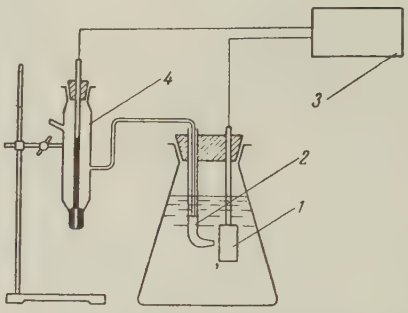

Fig. 1. Diagram of the set-up for measuring electrode potentials. 1) Experimental sample; 2) electrolytic jet; 3) potentiometer; 4) calomel electrode.

The oxygen in the water was determined according to Binkler, hydrogen sulfide—by the iodometric method, and pH—potentiometrically with a quinhydrone electrode. The electrode potentials of stainless steels are shown in reference to the normal hydrogen electrode.

1. The effect of Vibrio desulfuricans. As is well known, V. desulfuricans is an anaerobe (Krasil'nikov, 1949; Rubenchik, 1947). Therefore, the experiments were carried out in hermetically sealed flasks. However, the authors were not able to find concrete figures in the literature showing the effect of oxygen concentration on the growth of these bacteria. Meanwhile,

Table 1. The Chemical Composition of Steels 1Kh18N9T and 1Kh13 (GOST 5632-51)

Brands of steel	Elements, %							
	C	Mn	Si	S	P	Cr	Ni	Ti
1X18H9T	≤0.12	≤2.0	≤0.8	≤0.03	≤0.035	17–20	8–11	≤0.8
1X13	≤0.15	≤0.6	2–3	≤0.03	≤0. 03	8–10	≤0,6	—

this question is of prime significance in the study of the effect of these bacteria on the corrosion of metals in sea water occurring as a function of a number of conditions as oxygen concentrations change from values close to zero (under conditions of narrow clearances, for example) to the normal concentration of 7–10 mg/liter.

The experiments showed that, in hermetically sealed vessels, an initial concentration of oxygen in the range of 0 to 5 mg/liter had little effect on the vital activity of V. desulfuricans (Table 2). Thus, at an oxygen concentration of 1.1 mg/liter, the number of bacteria constituted 229 thousand per 1 cm^2, and H_2S was 33.2 mg/liter; at an oxygen concentration of 3.8 mg/liter, the number of bacteria constituted 286 thousand per 1 cm^2, while H_2S was 34.8 mg/liter. Similar results were obtained when $CaSO_4$, $MgSO_4$, and NaCOOH were added to the sea water (added according to the directions for Tauson's medium). The decrease of oxygen concentration to zero also led to no noticeable increase in the vital activity of the bacteria. The growth conditions for the bacteria changed markedly in the experimental flasks which communicated with the atmosphere. Here, an oxygen concentration of 7 mg/liter (this concentration is established in sea water under the influence of the atmosphere) sharply reduced bacterial growth as well as H_2S production.

When the effect of V. desulfuricans on the electrode potential of steel was studied, the oxygen concentration was 1.2–1.4 mg/liter. This concentration of oxygen was established in sea water after it was sterilized in the autoclave.

Experiments showed that sulfate-reducing bacteria have a strong effect on the electrode potential of stainless steels, shifting it sharply to the negative side. It is seen from Fig. 2 that the change in electrode potential of steel 1Kh13 due to the effect of sulfate-reducing bacteria constituted 150 mv. An even more significant change in the potential was observed when Na_2S was added to the flask, which caused an increase in the vital activity of these bacteria. In this case, the shift of potential in the negative direction reached 200 mv on steel 1Kh18N9T (Fig. 3). The character of the curves of changes in potential with time showed that hydrogen sulfide dissolved in water had little effect on the electrode potential. While the hydrogen sulfide concentration increased continuously (Table 3), the change in electrode potential was of a periodic character. The processes taking place on the surface of the steel as the result of the vital activity of bacteria are apparently of decisive significance. The number of bacteria, amount of hydrogen sulfide, as well as the pH value at the end of the experiment (after 5 days) on the measurement of electrode potentials are shown in Table 4.

2. The effect of Pseudomonas fluorescens liquefaciens. As is well known, P. fluoresens liquefaciens reduces nitrates to nitrites, and even to free nitrogen (Krasil'nikov, 1949). The average nitrate nitrogen content in the surface layer of the Black Sea constitutes 71 mg/m^3 (Chigirin and Danil'chenko, 1930). In connection with the fact that nitrogen activates metal surfaces (particularly chrome) (Akimov, 1945), it might have been expected that when stainless steels were placed into sea water containing this bacterial culture, the electrode potential would shift to the neg-

Table 2. The Effect of Oxygen on the Growth of Vibrio desulfuricans (period of growth 10 days)

Medium	Sea water						Sea water with additions of $CaSO_4$, $MgSO_4$, NaCOOH				
O_2 mg/liter at start of experiment	1.1	1.6	2.4	3.0	3.8	7.2*	1.0	3.6	4.2	4.9	7.0*
No. of bacteria in thousands/1 cm^2	229	342	296	331	286	129	846	908	1101	921	124
H_2S mg/liter at end of experiment	33.2	23.2	33.2	29.9	34.8	2.1	38.1	51.5	61.4	53.1	2.3

*Experimental flasks communicate with atmosphere (stoppered with sterile cotton plugs)

Table 3. The Change with Time of the Number of Sulfate-Reducing Bacteria and the Amount of Hydrogen Sulfide in Sea Water

Indicators	Initial amount	After 7 days	After 15 days	After 25 days	After 30 days
Number of bacter in thousands per 1 cm^2	100	318	713	781	760
H_2S, mg/liter	0,0	10,0	34,8	39,0	39,6

ative side in the process of denitrification. The results of experiments conducted along these lines are shown in Fig. 4.

As seen from our experiments, during the intensive growth of P. fluorescens liquefaciens in sea water, the electrode potential of steel 1Kh18N9T became 100 mv more negative after 48 hours (curves 1 and 2) as compared with the electrode potential of the same steel in the control (curve 3).

It should be noted that no noticeable change in the oxygen content of the water occurred during the cultivation of these bacteria either in hermetically sealed flasks or in flasks with cotton stoppers. Consequently, in the presence of bacteria in the sea water, the electrode potential of steel 1Kh18N9T does not depend on whether the experiments are conducted in hermetically sealed flasks or in flasks communicating with the atmosphere (Fig. 4: 1, 2).

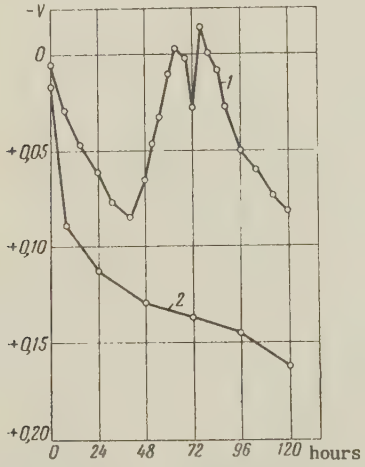

Fig. 2. The effect of Vibrio desulfuricans on the electrode potential. 1) Steel 1Kh13 with bacteria; 2) control.

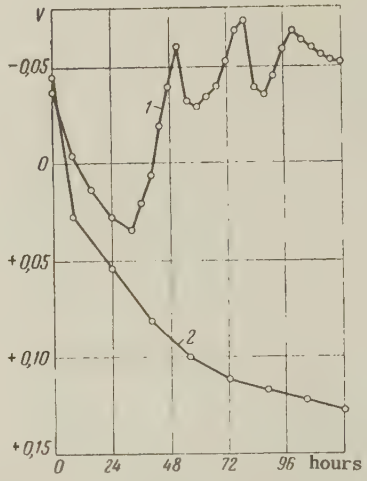

Fig. 3. The effect of Vibrio desulfuricans on the electrode potential. 1) Steel 1Kh18N9T with bacteria; 2) control.

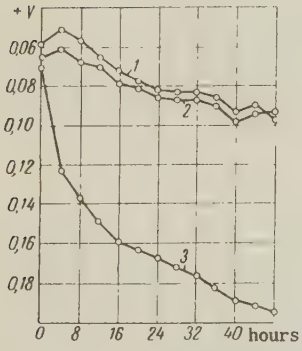

Fig. 4. The effect of Ps. fluorescens liquefaciens on the electrode potential of steel 1Kh18N9T. 1) With bacteria, in a hermetically sealed flask; 2) with bacteria, flask open to the atmosphere; 3) control.

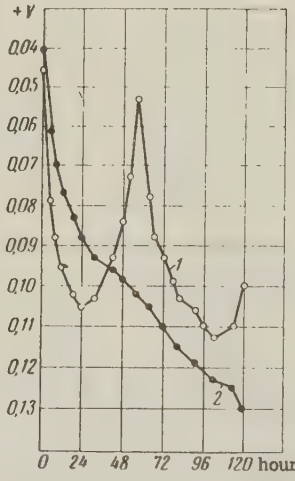

Fig. 5. The effect of L. crussa on the electrode potential. 1) Steel 1Kh18N9T with bacteria; 2) control.

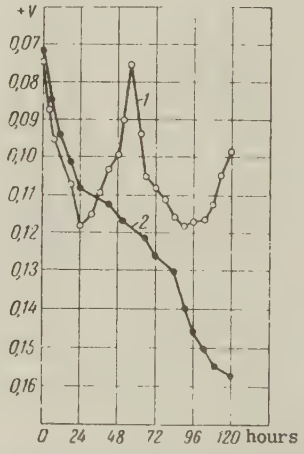

Fig. 6. The effect of L. crassa on the electrode potential. 1) Steel 1Kh13 with bacteria; 2) control.

Table 4. The Effect of the Vital Activity of Vibrio desulfuricans on the Hydrogen Sulfide Content and pH Value

Brand of steel	No. of bacteria in thousands per 1 cm_2	H_2S, mg/liter	pH
1X13	543	22,9	8,0
1X18H9T	962	37,6	8,0

Table 5. The Effect of the Vital Activity of Pseudomonas fluorescens liquefaciens on the pH Value (duration of experiment 48 hours)

Experimental conditions	No. of bacteria per 1 cm_2	pH	
		at start of expt.	at end of expt.
Flask communicating with the atmosphere	1686	7,72	7,68
Hermetically sealed flask	1738	7,81	7,64

As seen from Table 5, no difference could be noted in the conditions of cultivation of P. fluorescens, since after 48 hours the number of bacteria in both flasks was similar. The same table shows that practically no change in pH value was observed under the influence of P. fluorescens.

3. The effect of Leptothrix crassa. Experiments showed that activation of the surface of stainless steel takes place under the influence of the vital activity of L. crassa, i.e., the protective properties of the passive film are disrupted to some extent.

Figure 5 shows the change in electrode potential of steel 1Kh18N9T in a culture of L. crassa in sea water (curve 1). In the control, the electrode potential shifted in the positive direction during the course of the entire experiment. In the experiment with bacteria, in the first 24 hours, the electrode potential changed in the same way as in the control samples. Then, the potential periodically shifted sharply to the negative side. Under the given conditions, the difference in electrode potential values between the samples inoculated with bacteria and the control reached 60 mv. A similar phenomenon was also observed in experiments with steel 1Kh13 (Fig. 6). Measurements of oxygen concentration at the start and end of the experiments showed that the growth of L. crassa was accompanied by a decrease in the amount of oxygen (Table 6).

In the given case, however, the shift in the potential to the negative side which was noted can not be explained by a decrease in oxygen concentration. It is seen from Table 6 that in experiments with steel 1Kh18N9T, the oxygen concentration decreased from 6.2 to 4.7 mg/liter, while in the experiment with steel 1Kh13, it decreased from 5.8 to 4.0 mg/liter. This reduction in oxygen concentration caused a shift in the potential to the negative side only by a value of the order of 10 mv (Fig. 7), while in experiments with L. crassa, the drop in potential reached 60 mv.

The slight decrease in the pH value observed in the experiments had an insignificant effect on the electrode potential (Ulanovskii and Korovin, 1958).

Table 6. The Effect of the Vital Activity of Leptothrix crassa on Oxygen Content and pH Value (duration of experiment 120 hours)

Brand of steel	No. of bacteria in thousands per 1 cm_2	O_2, mg/liter		pH	
		at start of expt.	at end of expt.	at start of expt.	at end of expt.
1X18H9T	234	6.2	4.7	8.02	7.54
1X13	513	5.8	4.0	8.06	7.62

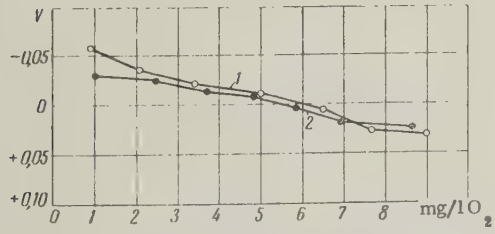

Fig. 7. The effect of oxygen concentration on the electrode potential. 1) Steel 1Kh13; 2) steel 1Kh18N9T.

SUMMARY

1. The effect of the anaerobic bacterium Vibrio desulfuricans and the aerobic bacteria Pseudomonas fluorescens liquefaciens and Leptothrix crassa on the electrode potential of stainless steels has been examined.

2. During intensive growth, these bacteria cause a shift in the electrode potential of stainless steels in the negative direction.

3. The shift of the electrode potential toward the negative side on individual areas of the steel surface indicates the disturbance of the protective passive film to some extent and creates favorable conditions for the rise of galvanic couples.

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PRESERVATION OF VIABILITY OF THERMOPHILIC ACTINOMYCETES AFTER LONG STORAGE

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In studying pure cultures, one may question the preservation of their viability under laboratory conditions. In this connection one must define the frequency of transfer and conditions for preservation of a typical culture.

A. I. Korenyako (1954) investigated preservation and properties of mesophilic actinomycetes dried at room temperature. There are only occasional references in the literature to the preservation of pure cultures of thermophilic actinomycetes. Schutze's observations (1908) indicate death of most spores in cultures of fully developed thermophilic actinomycetes preserved for several months at room temperature. In studying the effects of subminimal temperatures on hyphae of thermophilic actinomycetes, Novack (1912) established that they die off, when kept on agar and in hay infusion, in 14 to 18 days at 5 to 11 deg, in 27 to 30 days at 15 to 17 deg, and in 32 to 35 days at 20 to 21 deg. Cultures grown at different temperatures did not vary in their resistance to relatively low temperatures.

Lieske (1921) used samples of corn cobs, hay, and soil kept for prolonged times in sterile containers. Thermophilic actinomycetes, which developed after inoculation of the samples, died out shortly after their isolation as pure cultures (regardless of formation of aerial spores). Pure cultures having a minimum growth temperature of 40 deg and isolated from corn kernels preserved for 3 years, died within 8 days at room temperature. Thermophilic actinomycetes from garden soil, isolated at 60 deg, lost their viability within one to two months at room temperature. Pure cultures obtained from soil samples preserved for four months in a refrigerator, died out within one to five weeks when maintained at this temperature.

Erikson (1952) found that pure cultures of thermophilic actinomycetes die off within a week at room temperature; however their viability is preserved for 6 months in test tubes at 2 deg.

Rapid loss of viability by pure cultures of thermophilic actinomycetes is indicated by all of the above examples, despite certain contradictions. A common shortcoming of these studies is the omission of the detailed description of conditions of preservation of the cultures.

During 1950–1953 we isolated and studied pure cultures of thermophilic actinomycetes and their antibiotic properties (Kosmachev, 1956). Taking into consideration the data regarding the loss of viability of pure cultures of thermophilic actinomycetes, we carried out certain special observations. Twenty-three different cultures (Nos. 385, 391, 509, 534, 563, 589, 648, 674, 706, 707, 709, 717, 728, 806, 807, 809, 1067, 1360, 1363, 1366, 1367, 1393, 1400), containing representative species of Actinomyces and Micromonospora, were isolated on agar slants of the following composition: KNO_3–1 g, $(NH_4)_2SO_4$–1 g, Na_2HPO_4–1 g, $MgSO_4$–0.5 g, $FeSO_4$–0.001 g, chalk–4 g, 30% yeast autolyzate–15 ml, starch–20 g, agar–20 g, water–1 liter. After cultures were fully developed at 55 deg (2–3 days), the test tubes were placed in cardboard containers at room temperature. Agar dried completely in all experimental test tubes and was covered with a dry, powder-like film of spores. Every one of the 24 cultures grew well when transfered to the same medium half a year later. Thereafter we always used this simple method for the preservation of pure cultures of thermophilic actinomycetes. In this manner good growth was obtained when cultures were transferred after one year of storage.

By means of the above described procedure three pure cultures of thermophilic actinomycetes, of which two (Nos. 226 and 1754) were identified as different strains of Microspora vulgaris and one (No. 1869) as Actinomycetes sp., were preserved in test tubes at room temperature without transfer from 1951 to 1959. These cultures were studied by us in detail from 1951 to 1953. Culture No. 226, of particular interest to us, produced an antibiotic active against Staphylococcus aureus, Mycobacterium vadosum, M. tuberculosis var. avium, Bacillus brucella bovis. The antibiotic, obtained in a concentrated impure form, did not exhibit local toxicity. Culture No. 1869 indicated antibiotic activity against S. aureus and Bacterium coli. Culture No. 1754 inhibited growth of S. aureus and M. vadosum. Spores of the three cultures preserved from 1951 to 1959 showed good growth at 55 deg when placed on corn agar, as well as on NPA and on synthetic agar. Cultures revived after eight years maintained their thermophilic, morphologic, cultural, and physiological characteristics. Of particular importance is their maintenance of antibiotic activity, inasmuch as it has been established that this property may be lost or considerably weakened by numerous transfers (Krasil'nikov, 1950). Cultures grown in 1951 are being preserved. There is no reason to assume that viability of spores of pure cultures of thermophilic actinomycetes is restricted to 8 years.

Establishment of the prolonged preservation of viability, as well as morphologic, physiologic, and antibiotic properties of the cultures in question, contraindicates the necessity of frequent transfers for their

preservation in a collection of pure cultures. The establishment of similar conditions for thermophilic actinomycetes and other organisms not evaluated in this respect, will allow not only elimination of numerous transfers of cultures, but will probably insure better preservation of the original antibiotic and other properties.

SUMMARY

1. Pure cultures of some thermophilic actinomycetes are well preserved at room temperature in test tubes on dried synthetic agar.

2. Three pure cultures of thermophilic actinomycetes belonging to genus Micromonospora and to genus Actinomyces, when stored during 8 years in the manner described above, preserved their viability and their morphological, physiological, and cultural characteristics, as well as the original antibiotic activity.

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A NEPHELOMETRIC METHOD FOR DETERMINING PROTEINASE ACTIVITY OF BACILLUS SUBTILIS

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Various methods of determining proteolytic activity have been described in the literature.

The separate titration of peptides and amino acids with an alkali in alcohol has been widely used (Grossman, Heyde, 1929). This method, however, admits of subjective errors as the color of the indicator does not change distinctly enough. For serial analyses, under conditions of production of fermenting preparations, as well as for the industrial application of proteinase of bacterial or fungoid origin, the different methods of titration—in the presence of acetone (Lindertrom-Lang, 1927), ninhydrin (Van Slyke, McFadyen, Hamilton, 1941), and formalin (Sorensen 1907)—have proved to be unsuitable. The gasometric method of determining the carboxylase or amine groups of amino acids requires a special apparatus and delicate manipulations (Van Slyke, 1912, 1913, 1929; Van Slyke et al., 1941).

For theoretical purposes, when it is necessary to determine the depth and to analyze the nature of the digestion products of the substrate, the spectrophotometric and colorimetric methods and their modifications, in which the Folin-Ciocalteau reagent is used (Anson, 1939, Laury et al., 1951), have been found to give good results. Recently the method of measuring the optical density of tyrosine in transparent hydrolyses at 275–280 mμ (Northrop et al., 1948; Hagihara, 1954; Hagihara et al., 1958) has attracted attention. Nevertheless, for determining the proteinase activity of Bacillus subtilis in a colored culture medium many of the colorimetric methods have proved to be inconvenient.

The viscosimetric methods (Imshenetskii and Kasatkina, 1954; Koch, Ferrari, 1958) which are frequently used for determining the proteinase action do not test the effect of the color of the culture medium.

Kunitz (1935) proposed a method for measuring the proteinase activity of B. subtilis by the time it takes to clot milk. This method is widely used in the Carlsberg laboratory (in Denmark) by Guntelberg (Guntelberg and Ottensen, 1952).

For conditions of industrial production, a method for determining enzyme action which does not require a special substrate is necessary. A substrate commonly used for testing the activity of the majority of proteinases is gelatin.

MATERIALS AND METHODS

In the present tests a crystalline preparation of proteinase of the B. subtilis from the Japanese firm "Nagase" was used together with the culture medium of this strain which was cultivated in laboratory conditions on a nutrient medium containing siftings. The culture medium was a deep brown color. The gelatin solution (0.1%) was prepared in the following way: 100 mg of gelatin were left to swell in 2–3 ml of water for 10 min and then hot M/15 phosphate buffer pH 7.17 was added gradually to dissolve the gelatin. The solution was then transferred to a measuring retort with a capacity of 100 cc. Before incubation the gelatin solution was kept in a thermostat at 40 deg. Before its incubation with gelatin the enzyme solution was processed on ice.

Determination of Proteinase Activity.

0.2 ml of enzyme solution of the appropriate dilution were poured into a series of tubes (maximum = 9). The dilution of the enzyme solution or culture medium must be so chosen that the inhibition values fall within the range 0.100–0.300 of the drum scale of the nephelometer. The tubes were transferred to a water thermostat with a temperature of 40 deg ± 0.5 deg. After 5 min 0.8 ml of the gelatin solution was added to the first tube and the time noted. At one minute intervals the gelatin solution was added to each succeeding tube. After a 10 min incubation period, 4 ml of 20% solution of trichloroacetic acid was added to the tubes, which were then left at room temperature for an hour and then subjected to a nephelometric analysis with a photoelectric colorimeter FEKN-57 using green filter No. 10 and 3 mm bulbs. The contents of the tubes must not be stirred before the nephelometric analysis as the dimensions of the precipitated particles decrease, thereby increasing the transparency of the medium, and this may heighten the results. As control we used a mixture of 0.8 ml of gelatin solution and 0.2 ml of distilled water. A solution of a boiled enzyme cannot be used as control as it is comparatively thermally stable whereas the amount of the precipitate from the fermenting protein was so small as to be beyond the limits of sensitivity of the nephelometer. After calculating the difference between the inhibition values of the control and tested samples, the highest gelatin concentration expressed in micrograms of nitrogen was calculated from the calibration curve (Fig. 1). To obtain the calibration curve 0.1% solution of nutrient gelatin was poured in ascending order (0.1–1.0 ml) into 10 tubes. Distilled water was added to bring it up to 1 ml and then 4 ml of 20% solution of trichloroacetic acid was added. A nephelometric analysis and nitrogen determination by the Kjeldahl method were carried out after an interval of 1 hour.

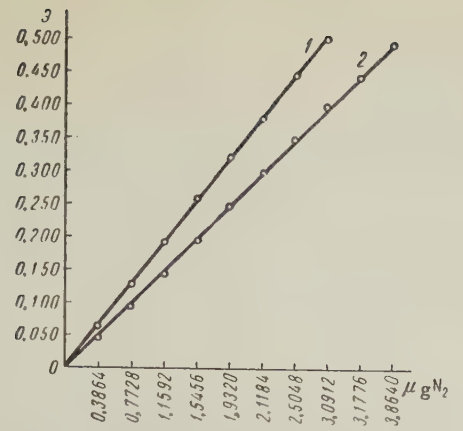

Fig. 1. Calibration curve of gelatin concentrations. 1) Blue light filter; 2) Green light filter.

Figure 1 shows that the dependence of light absorption on gelatin concentration has a linear character. As distinct from the curve obtained when a blue filter was used, the curve produced with a green filter is plotted on the basis of complete proportionality between the gelatin concentration and the optical density values.

We investigated next the problem of the kinetics of enzyme action—the selection of the duration of enzyme action under conditions of those gelatin concentrations which were determined proportionally on the calibration curve. For this purpose 8 ml of 0.1% gelatin solution and 2 ml of enzyme solution of an appropriate dilution were poured into a tube. At predetermined intervals of time 1 ml of the medium under investigation was added to each tube containing 4 ml of 20% trichloroacetic acid solution (Fig. 2).

Figure 2 shows that the enzyme action during 3 minutes has a linear character; thereafter a bend appears in the curve (see Curve 1). In studies concerned with theoretical aims, it is possible to use this method without special kinetic amendments if the enzyme action lasts for 3 minutes. Nevertheless, for the sake of convenience in work of mass analyses,

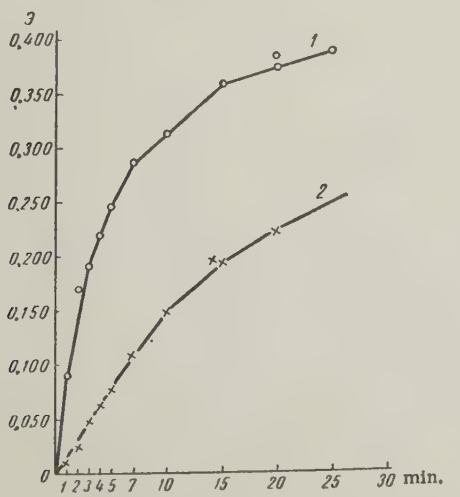

Fig. 2. Change in proteinase activity in relation to time. 1) Proteinases 2.0 g/ml; 2) 0.5 g/ml.

Table 1. Effect of Enzyme Dilution on Its Action

Inhibition	Enzyme concentration				
	control	4	2	1	0.5
Average inhibition	0,388	0,131±0.008	0.240±0,016	0.304±0,003	0,347±0.006
Difference in inhibition	—	0.257	0.148	0.084	0.041

we took the incubation time to be 10 minutes. The duration of enzyme action represented by the linear part of the curve increases in proportion to the dilution of the enzyme solution (see Curve 2).

Lastly we investigated the effect of the dilution of the enzyme on its action for a specific period of incubation (10 min.). The results are shown in Table 1.

From the figures given in the table it will be observed that a twofold dilution of the enzyme results also in an approximately twofold decrease in the inhibition value. The average fluctuation in the inhibition of similar specimens is within the range ± 0.004–0.005.

The proteinase activity is expressed in units of enzyme action by the following formula:

$$PA_{gel}\ \frac{(C_i - E_i)\ 60 \cdot 5 \cdot D}{10 \times 1000},$$

where PA_{gel} is the proteinase activity (gelatin substrate); C_i is the inhibition of the control specimen; E_i is the inhibition of the experimental specimen; and D is the dilution.

The difference in the inhibition values $C_i - E_i$ is expressed in micrograms of nitrogen on the calibration curve.

Unit of enzyme activity is defined as that amount of enzyme which catalyzes hydrolysis of gelatin to produce 1 mg of nitrogen in an hour at 40 deg.

The described method has been verified by mass analyses carried out in the course of the determination of proteinase activity of the culture medium of B. subtilis and can be recommended for the determination of enzyme activity in a highly colored medium.

SUMMARY

1. A nephelometric method of determining proteinase activity is proposed.

2. In accordance with this method, a provisional equation of a unit of enzyme activity has been adopted.

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STRUCTURE OF YOUNG AZOTOBACTER COLONIES

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Colonies of capsulate bacteria are characterized by a definite microscopic structure, peculiar to the particular species and related to the way in which the microbes move during their multiplication.

El'bert (1928) described five basic types of structure of young colonies of capsulate bacteria: I–concentric, II–concentric-diffuse, III–stellate, IV–ansiform-scalloped, V–aciculate.

The literature contains no information on the structure of colonies of different species of Azotobacter; there are only descriptions of the morphology of colonies and microcolonies of Azobacter chroococcum in the works of Bachinskaya (1935), Rybalkina (1938), Novogrudskii (1937), and Lohnis (1905). However, not one of these authors dwells on the arrangement of the cells in the colonies.

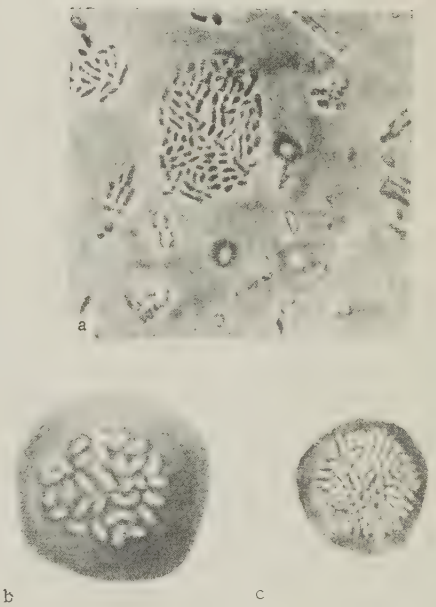
In view of the importance of this morphological character for the discrimination of capsulate bacteria we made a study of the microstructure of young colonies of the main species of Azotobacter. The investigation was made by the method of agar microscopy, devised by El'bert (1928) for bacteria of the capsulate group.

METHODS

One or two drops of liquid culture of Azotobacter were smeared with a spatula or loop over the surface of agar in a Petri dish. The two halves of the dish had previously been dried to remove condensation water. The inoculated plate was installed in an incubator until the first traces of growth appeared. Then, from a region where growth was noted we cut out an agar wafer 1.5 2 cm in area with a heated lancet and mounted it on a slide. The mount prepared in this way was examined under low power at first, then under high power (objective × 40, eyepiece × 15). For illumination of the specimen we used a strong light source (100 cp lamp), and the sharpness of the image was adjusted by means of the diaphragm.

By this method we examined 34 Azotobacter strains isolated from different regions of the USSR (three stock strains–A. chroococcum–53, A. agile–2, A. vinelandii–1; 18 local strains isolated from soil, water and mud, including nine strains of A. chroococcum, seven of A. agile, and two of A. vinelandii; 11 strains of A. chroococcum, obtained from the Institute of Pedology of the Academy of Sciences Kazakh. SSR, and isolated from the soils of Akmolinsk, Kokchetav and Alma-Ata regions; three strains of A. chroococcum, A. agile and A. vinelandii from the Armenian SSR). The structure of the colonies was studied from the time of their formation (eight hours old) until the transition from the rodlike form to the coccus.

We had occasion to examine A. chroococcum in particular detail since the structure of colonies of this species is characterized by some morphological diversity. The formation of the colonies in favorable conditions begins when the culture is approximately eight hours old. At first the cells are arranged concentrically, after which radial series appear, the colony becomes surrounded by a chain of cells, and after 20 hours growth it has a fully-developed radial-concentric structure (Fig. 1, a). The colony consists of diplobacilli lying close to one another. After some time stratification appears in the colony, and this is very distinct after 28–30 hours growth; after 36–40 hours the bacilli change into the coccoid form, the appearance of cocci beginning in the center of the colony, while the margin still consists of rodlike cells, and it is not until two to three days later that all the cells assume the coccoid form, and the growth of the colony then terminates.



Twenty-hour growth of Azotobacter colonies (photomicrograph 1500). a) Azotobacter chroococcum; b) Azotobacter agile; c) Azotobacter vinelandii.

Hence, colonies of A. chroococcum are characterized by a radial-concentric structure with the subsequent appearance of stratification.

A. agile colonies have quite a different structure. Here we find a great formation of mucus, which comprises the convex base of the colony. The cells are much sparser than in the A. chroococcum colony, but form a definite pattern, typical of the stellate-diffuse type of structure (Fig. 1, b).

In A. vinelandii the cells are arranged radially from the very start of colony formation and retain this arrangement throughout the development of the colony. Young colonies have a clearly defined circular form (Fig. 1, c).

Thus, in the structure of the young colonies of different Azotobacter species there are substantial differences, and from these their specific affinity can easily be determined, and a pure culture can be isolated from a mixed one. In their type of structure young Azotobacter colonies differ markedly from other species of microorganisms of soils and muds.

SUMMARY

1. From a study of the microstructure of young Azotobacter colonies three main types of structure have been found. A. chroococcum is characterized by a radial-concentric type of structure, A. agile by a stellate-diffuse type, and A. vinelandii by a radial type. These differences in colony structure can be used as additional cultural characters for discriminating Azotobacter species.

2. From the nature of the structure of young colonies the growth of Azotobacter in a mixed culture can easily be detected.

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MICROBIOLOGY—YESTERDAY AND TODAY *

Selman A. Waksman

"And in today already walks tomorrow"
Schiller

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It is difficult to speak, except in very relative terms, of time and space. Who can tell exactly when today will end and tomorrow begin, since, after all, tomorrow is merely a continuation of today. It is just as difficult to establish exactly where and when yesterday ended and today began. This is true not only of cosmological phenomena and of broad historical periods, but also of human ideas and experiences, with all the overlapping of personalities, concepts, and factual observations involved.

In attempting to trace an important scientific discovery, it is not always easy to credit those who have conceived a certain idea and to trace the various stages of development through which it progressed until it culminated in a fundamental scientific or practical contribution to human knowledge. It is often virtually impossible to credit a particular scientific contribution to one individual investigator, without giving due consideration to those who preceded him, to those who assisted him, and often also to those who followed him.

The popular mind likes to associate a particular period rich in scientific discoveries with a single individual, crediting him with all the accomplishments of the period and completely disregarding numerous others, without whose contributions such attainments would have hardly been possible. Thus a Newton and an Einstein, a Kepler and a Galvani, a Pasteur and a Koch have worn the laurels of scientific accomplishments to which many others have made important contributions, in the form of ideas or of actual observations. No wonder then that one encounters occasional dissatisfaction with generalizations of this nature, especially when counterclaims to certain scientific observations are based on national feelings and attitudes. No better illustration could be presented than an analysis of some of the early contributions to the field of microbiology.

It is important to attempt the correlation of changes, phases and attitudes in microbiology, since in general there has been a gradual shift in recent years from the endeavors of the individual investigator to those of a team. This shift in emphasis, the difficulties of associating particular eras in a field of science with its eminent representatives and the influence of subsequent developments upon the progress in this field, can best be illustrated by reference to the pioneer investigators in microbiology. These men may be regarded as having set the pace for the science of yesterday.

Early Investigators in General Microbiology.

Certain outstanding names in microbiology may be selected to elucidate the changing concepts in the development of this particular field of science. Does the death, 30 years ago, of Wilhelm M. Beijerinck, the great pioneer of yesterday, or, only six years ago, of Sergei N. Winogradsky, the last of the older giants of microbiology, signify that microbiology of yesterday was brought to an end with the passing of one or the other Does it mean that the latter merely survived physically most of his contemporaries, and that the microbiology of today long ago overtook him and moved into a new era Does the sudden death of Albert J. Kluyver, one of the outstanding investigators of recent years, signify that microbiology of today has come to an end and that we must start looking for the microbiology of tomorrow Or was Kluyver merely a symbol of certain ideas that have been dominating many of the recent concepts in microbiology, and that the modern period as such is far from coming to an end Should one be so bold as even to attempt to construct a bridge between the developments in microbiology that took place yesterday and those that are occurring before our very eyes today

Let us attempt to analyze briefly the microbiology of yesterday. It is essential, first of all, to examine the methods which were characteristic of that period and by which outstanding results were obtained. Basically, the methods were characterized by observations of the occurrence of microorganisms in nature, by the isolation of these organisms in a pure state, and by the description of many new forms, thus firmly establishing the ecological and botanical aspects of the science of microbiology. In many instances, this accomplishment was not a result of "field studies," so familiar in the historical development of the older biological sciences. The microbiologists of yesterday were largely concerned with a search for organisms responsible for certain known processes, rather than for mechanisms and reactions carried out by such organisms.

It all started with the observations of Anton von Leeuwenhoek and the other microscopists. Microbiology began to make rapid progress about the middle of the last century. It became definitely recognized as a field of science through the work of the zoologist

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Christian G. Ehrenburg, the botanist Ferdinand Cohn, the mycologist Heinrich A. DeBary, and others. Their work was limited primarily to descriptions of new organisms and to the establishment of well-recognized genera and species. The elucidation of the chemical processes brought about by these and other microbes, and of their role in fermentation and in the causation of disease was started with the work of the chemists E. A. Mitcherlich and Louis Pasteur.

A definite forward step in the study of microbes came soon through the pure culture studies of Robert Koch, as well as the contributions to our knowledge of the association of specific organisms with known diseases, largely through the work of Pasteur, Koch, and numerous others. The infectious nature of disease thus became gradually elucidated. This development was soon followed by the investigations of Paul Ehrlich in the field of chemotherapy, especially the use of dyes and arsenicals in the treatment of infectious diseases, and of Emil von Behring and Shibasaburo Kitasato, on antitoxins and vaccine therapy. These contributions broadened our concept of infectious diseases and their control.

Simultaneously came the biochemical studies of Winogradsky and Beijerinck on the role of various bacteria in important natural processes, such as nitrification, nitrogen fixation, and sulfur oxidation. These were followed by the work of numerous other microbiologists who, in search for organisms responsible for known processes, concerned themselves with the study of the occurrence, nature, and activities of various groups of microbes. The knowledge thus gained served to broaden further our concepts of general microbiology and of the relation of microbes to natural processes, especially those important in human economy. If one were to mention also D. Iwanowsky's and Beijerinck's work on viruses, Joseph Lister's ideas on disinfection, Woronin's, DeBary's and Burril's work on plant pathogens, one would merely touch upon some of the highlights of the contributions to our knowledge of the role of microorganisms in natural processes, which characterized the classical microbiology of yesterday.

It has been said time and again that early microbiology suffered from its emphasis on practical problems. Pasteur was more interested in the processes of fermentation of beer and wine, in the diseases of the silkworm, in the causation and control of rabies and anthrax than in the causative organisms themselves. It should be remembered, however, that during the same period Robert Koch was searching for the organism responsible for the causation of tuberculosis in man and in animals and for the life cycle of the anthrax bacillus; his contributions to methods of control of these diseases were rather secondary in nature. Beijerinck was concerned with the bacteria responsible for the processes that take place in soils and water basins, rather than with the processes themselves. Winogradsky was looking for bacteria capable of bringing about certain autotrophic chemical reactions and thus introduced a new concept into microbiology.

We need not add further to this list of investigators concerned with known processes, whether they be diseases of animals and plants, biological transformations in soils and in water basins, or important industrial fermentations. Other investigators who concerned themselves largely with the nature of these processes soon followed. They also devoted considerable attention to the methods of control of microbial activities. These involved a variety of problems, ranging from the selection of pure cultures for a particular fermentation to the establishment of principles of chemotherapy, which gradually led to the control of the growth of injurious microorganisms. Before we proceed, however, to a further comparison and analysis of the concepts and contributions of the microbiologist of yesterday and today we should pause here to discuss briefly the contributions of other scientific disciplines to microbiology. This science which owed much of its origin to the information and techniques of other fields appears to repay its debt now in a most efficient manner by furnishing invaluable information, model systems, and technical tools to other sciences.

The Older Sciences and the Advance of Microbiology.

One must consider first of all the contributions of the general biologists, especially zoologists and botanists, who were the first to offer in many universities courses in bacteriology. The chemist came next with his contributions to our knowledge of the nature and nutrition of microbes. Among those who have made extensive use of the rapidly increasing knowledge of microbes and who have themselves contributed greatly to it, the clinician also occupies a prominent place; he was among the first to recognize the great potentialities of this new field of knowledge. If Cohn is to be considered as the representative botanist and Pasteur as the chemist, certainly Koch and Lister would personify, in the very best tradition, the clinicians.

Next must be considered the work of a number of other scientific workers concerned with the nature and activities of microbes. The veterinarian, the plant pathologist, the agronomist, the soil investigator, the sanitary worker, the food expert, the brewer and the distiller, and finally the industrialist interested in fermentation products, ranging from antibiotics to organic acids, have all left their imprint upon microbiology.

Very few of those who are concerned with microbes today, in a period when biochemistry is supreme and when clinical applications appear to have reached their zenith, appreciate the important contributions of the botanist to microbiology. Those who first isolated, identified, and described microbes, whether bacteria or actinomycetes, fungi or viruses, laid a solid foundation for subsequent growth of microbiology. It is the botanist who deserves all the credit for this work and it is he who must shoulder most of the blame for the fact that the medical workers took over rapidly the study of the bacteria, so that they soon came to be recognized as the leaders in the field. It is often forgotten that the virus-host relationship as well was first established by the botanists.

The chemist was also among the first to recognize the great potentialities of microbes as chemical, or rather biochemical, agents. Beginning with Pasteur's work on the separation of tartrates by molds and the role of lactic and butyric acid bacteria as agents of fermentation, there followed a long sequence of chemical investigators, who studied the production of alcohols, organic acids, vitamins, and finally antibiotics. The selection of various microbial strains and the development of the microscope and the ultramicroscope resulted in new techniques for the study of bacteria, viruses, and other groups of microorganisms. The chemist was responsible for the development of methods used in the study of the composition of microbial cells, of enzyme systems, and the isolation of viruses. The chemist, beginning with Paul Ehrlich, followed later by Michael Heidelberger, Gerhard Domagk, and Jacques Trefouel, initiated and developed chemotherapy. The chemist has often utilized the microbe as a model for the investigation of reactions taking place in the metabolism of higher forms of life.

The Microbiologist of Yesterday.

Let us now compare the microbiologist of yesterday and today and the approaches and problems with which each has been concerned.

To elucidate some of the general ideas and scientific contributions of the microbiologist of yesterday, and to define the contributions made by the investigator to this field, we may best consider certain specific problems, in summary form.

First of all, attention was focused upon the methods needed for the isolation and identification of bacteria and other microorganisms, the growth of these organisms in pure culture, and the determination of the role of these organisms in certain processes, such as causation of disease, spoilage of foodstuffs, or the numerous reactions taking place in the soil and in the sea. The life cycles of the organisms thus isolated often played an important part in these investigations. No wonder that the postulates of Koch, so characteristic of microbiology of yesterday, still dominate certain present-day concepts of our knowledge of the relationship of organisms to disease. These concepts, in various modifications, have also influenced our understanding of the role of microorganisms in biochemical reactions taking place in such natural substrates as soil and water.

The foregoing phases of the science were frequently followed and often accompanied by a search for the chemical reactions brought about by the various microorganisms. Almost simultaneously, a search was made for the means necessary to control the microbes responsible for these reactions. Thus, the studies of Pasteur on microbial fermentations and on the causation of rabies and the work of Ehrlich and Metchnikov on disease mechanisms and immunological reactions were followed immediately by efforts to control the organisms responsible for these processes so important in human economy and in human welfare.

Thus the history of microbiology of yesterday may be said to have embraced the following concepts.

Fermentation and Putrefaction. None of the contributions made by the chemist to microbiology were more significant than those dealing with fermentation and putrefaction. These concepts, largely enunciated through the classical studies of Pasteur, came to play an important role in the development of our knowledge of microbial nutrition and microbial biochemistry. Gradually the term "putrefaction" was dropped, and "fermentation" eventually acquired great significance. Pasteur's original dictum, "fermentation—c'est la vie sans oxygen," came to mean far more than the utilization of energy in the absence of oxygen, or merely anaerobic processes. Fermentation gradually came to signify metabolism of microorganisms, as a whole, and enzyme mechanisms responsible for metabolic reactions in particular. Today the term is being applied also to aerobic processes, in the absurd designation of "aerobic fermentation," and even to microbial life as a whole. Thus, side by side with respiration, fermentation introduced a new concept into life and the vital reactions upon which life in general and microbial life in particular are based.

Autotrophy. The original contributions of Winogradsky to our knowledge of sulfur bacteria, iron-oxidizing bacteria, and nitrifying bacteria gave birth to a new concept of bacterial life. It had to do with the ability of certain bacteria to utilize energy liberated in the oxidation of simple chemical elements and compounds. The role of microbes in natural processes thus gained added importance. Microbes were now no longer looked upon as mere "scavengers" and "pathogens"—they came to be recognized as biological systems on a par with other major biological groups, such as higher plants and animals, having broad relationships to both inanimate and animate nature.

Microbial Ecology. The influence of the environment upon the occurrence and activities of microorganisms came next into consideration. Among the problems involved were those concerned with the effect of the animal or plant host upon the invading parasite and with the significance of symbiotic relationships between plant and animal life, on the one hand, and microbial life, on the other. One must also recognize the problems concerned with the effect of the soil and atmospheric environment on the free-living and the saprophytic microorganisms. The previous concepts on aerobism vs. anaerobism, on autotrophy vs. heterotrophy, on parasitism vs. saprophytism, were soon to be followed by those of DeBary, M. Ward, and Beijerinck on symbiosis vs. antibiosis, and of Theobald Smith and others on insect vectors.

One may also consider, in this connection, the numerous investigations on the life cycles of bacteria. The earlier concepts on monomorphism and pleomorphism that occupied so much of the attention of the bacteriologists of the last century, beginning with the ideas and contributions of Ferdinand Cohn and of Karl W. Nageli—the chief corresponding protagonists of such concepts—were gaining momentum rapidly. They led to a broadening knowledge of bacteria as natural systems, with such contributors as Sigurd Orla-Jensen, and a better understanding of the life cycles of bacteria, notably through the work of A. T. Henrici. On the one hand, they led to the various systems of

bacterial classification, such as those of Migula, Chester, Lehmann and Neumann, Bergey, and Krassilnikov; on the other hand, they led to the later pleomorphists, notably F. Lohnis, Ralph R. Mellon, and H. Enderlein.

Biochemical Concepts. The growth and nutrition of microorganisms, in pure and in mixed culture, automatically attracted much attention. The development of synthetic and organic media for the growth of various microorganisms involved many problems of physiology and biochemistry. The work of Raulin and Czapek on the nutrition of fungi led to the eventual recognition of the importance of growth-promoting substances or vitamins and trace elements in the nutrition of many bacteria. This was exemplified by the work of Orla-Jensen, Lwoff, and numerous others. The investigations of O. Warburg, Michaelis, Meyerhof, Harding, Neuberg, Kluyver, and others led to a proper understanding of the mechanism of transformation of energy sources and of cell synthesis. The knowledge thus gained has contributed greatly to a better understanding of the nutrition of animals and man, and tended to bridge the gap between the concepts of microbiology of yesterday and that of today.

Disease and Chemotherapy. These problems received the greatest consideration by the microbiologists of yesterday. Numerous microorganisms were isolated, cultured, and tested for their effect on experimental animals. Their cells or cell products were used for immunization against infection, in the form of vaccines, and for therapy, in the form of serums and antitoxins. The ability of bacteria and viruses to bring about various immunological reactions in the blood of experimental animals opened new doors into the unknown living world. The sensitivity of microorganisms to various antimicrobial drugs was determined, in order to establish the efficacy of such drugs as potential chemotherapeutic agents. It was soon established that there is a striking parallelism in the activity of such drugs on bacteria in the test tube and in experimental animals.

The initial work of Lister and others on antiseptics and disinfectants, of Metchnikov on immunological reactions, and of Ehrlich on the synthesis of antimicrobial drugs embraced a series of scientific contributions to the field of microbiology that proved of inestimable value to human health. The antimicrobial potentialities of dyes, arsenicals, and mercurials, later followed by the sulfonamides and sulfones, finally led to the antibiotics that revolutionized medical science and medical practice.

Following the discovery of salvarsan, Ehrlich believed that chemical agents effective against true bacterial diseases were not far away, but a quarter of a century elapsed before the sulfa drugs came into being. There were certain important reasons for this long delay. Chief among these was the dominant idea of Ehrlich of a "maxima-sterilizans" which so impressed itself upon his followers that all their attention was centered upon bactericidal substances rather than upon bacteriostatic agents. The former were too toxic. The latter were either overlooked entirely or were found to have only a weak antimicrobial activity. There was also a lack of appreciation of the selective action of suitable antibacterial agents upon the parasite versus the host.

Saprophytic Microorganisms. Full recognition of the importance of microbes in the cycle of life in nature was centered around their activities in soils and in composts, in food and in food products, in water and in sewage. Some of the problems thus involved were characteristic of one particular substrate; others were more general. Among the problems on the borderline between parasitism and saprophytism, one may mention the production of bacterial toxins (botulinus), the problems of cellulose decomposition in textiles and paper, and the general damage to industrial and food materials.

Taxonomy and Descriptive Microbiology. Finally, one should not overlook the thankless labors of those who worked hard to make microbes recognizable. Beginning with the purely botanical investigations of Cohn, Wehmer, and others, and continuing through the work of such men as Chester, A. Mayer, Orla-Jensen, C. Thom, Bergey, Breed, and others, whether classified as bacteriologists, mycologists, protozoologists, virologists, or algologists, monumental contributions were made to our understanding of the microbes, their nature, and taxonomic relationships. Although this field is far from exhausted, as evidenced by the numerous new species of microorganisms, especially actinomycetes, still being described at this moment, it must be admitted that this process of codification of new genera and species is to be credited to the microbiologists of yesterday.

The investigators concerned with these various phases of microbiology of yesterday were primarily biologists, although they frequently made outstanding contributions to the biochemistry of microorganisms. Their approach to the problems involved was primarily biological. They were ecologists and physiologist, primarily concerned with cause and effect. Finally, they were interested in the methods of control of microbial life. They were concerned with microbial populations rather than with single cells. They were interested in broad biological activities rather than in specific biochemical reactions. It is impossible to conceive of a microbiologist of yesterday who would be voicing such comment as that made by one of the microbiologists of today that "Bacteria have no physiology, but they have a biochemistry."

The Microbiologist of Today.

To characterize the microbiologist of today, we must give first consideration to his use of the quantitative method and of the biochemical approach. It is frequently difficult to say with any degree of certainty where the work of the general biochemist leaves off and that of the microbiologist or the microbial biochemist begins. To emphasize the difficulty of designating investigators either as biochemists or as microbiologists, it is sufficient to cite such outstanding names as O. Warburg and O. Meyerhof, K. Lindestrom-Lang and Krebs.

New concepts have been introduced into microbiology, concepts hardly visualized by the microbiologist

of yesterday. For the sake of illustration, let us consider the genetic concept. Only yesterday, the genetics of bacteria was still a dormant or at least a highly controversial subject. From Mendel to Morgan and from Darwin to the modern evolutionists, the geneticist dealt entirely with higher plants or animals. Although some investigators were occasionally interested in the genetics of certain groups of microorganisms, as in the case of Blakeslee's work on the Rhizopus group, they would scarcely recognize the results of their efforts in the recent contributions of G. Beadle and E. L. Tatum, of J. Lederberg and G. Pontecorvo.

Recent developments in the fields of bacteriophages and of microbial enzymes are other illustrations that would characterize the endeavor of the microbiologist of today, as opposed to that of yesterday. The same is true of the investigation of metabolic reactions of one organism under the influence of the products of another. Microbial populations, when they were considered at all, are studied from the point of view of single cultures, with all the complex reactions involving problems of resistance and sensitivity, parasitism and saprophytism. No wonder, therefore, that the field of antibiotics, involving not only microbiological, but also chemical and biochemical considerations, could blossom forth today rather than yesterday. The growing knowledge of viruses and enzymes, of vitamins and antimetabolites, has added new aspects and new approaches to the microbiology of today. The microbiologist is now concerned with problems and with approaches that are distinctly different from those to which his predecessor, the microbiologist of yesterday, devoted his major attention.

The present-day interests in microbiology can be summarized briefly as follows:

Metabolism of Microorganisms. Although the earlier microbiologists were frequently concerned with problems of microbial nutrition and with the mechanism of transformation of substrate constituents, it is the investigator of today who is primarily interested in such questions as the role of specific chemical constituents of the substrate in the nutrition of microorganisms, the transformation of such constituents into other compounds, either intermediary or final in nature, and metabolic pathways in general. These investigations involve problems of oxidation and reduction, reactions of fermentation, and the various cycles of transformation involved in biosynthesis in general and the biogenesis of specific metabolic products, like antibiotics, in particular. The similarity of such biochemical reactions to those involved in parallel transformations in cells of higher forms of life has frequently been indicated. The availability of isotopes and of modern equipment has greatly facilitated study of the problems with which many microbiologists of today are concerned.

Enzymatic Mechanisms. The microbiologist of yesterday was primarily interested in those enzyme systems which involved questions of hydrolysis and synthesis, notably those involving transformation of proteins and amino acids, starches and sugars. The microbiologist of today is concerned with problems of enzyme adaptation, with enzymes of respiration and oxidation, and with numerous other enzyme systems undreamt of by the microbiologist of yesterday.

Genetic Problems. These involve problems of variation, mutation, and selection mechanisms, of genetic transfers and recombination, of resistance to antimicrobial agents, and the development of microbial strains with specific biochemical deficiencies. All of these have tended to transform completely our understanding of microbial life and of microbial nutrition, and have placed in the hands of the microbiologist new and effective tools. New vistas of cell growth and cell synthesis have also been opened.

Viruses and Phages. The chemical purification of viruses and phages, information on the mechanism of interaction of bacterial cells with phages, on the relations of prophage systems to mature phage, and finally the gradually evolving phage-gene concept, have thrown much new light upon the nature and activities of ultramicroscopic forms of life. They have also greatly enlarged our understanding of the relationship between host and parasite, between individual cells in complex populations, problems of reproduction, and a host of other phases of microbial life.

Formation of Growth-Stimulating and Growth-Inhibiting Substances. The formation of vitamins and antibiotics and the knowledge gained from the study of the action of these substances upon the growth of various groups of microorganisms have greatly enlarged our understanding of health and disease, and have provided tools for improving human and animal health and for extending the life span of man. Problems of sensitivity and resistance of microorganisms to antibiotics have contributed to a better understanding of microbial populations and the changes they are able to undergo, of problems of virulence vs. avirulence, and a host of others. Microorganisms have come to be recognized as ideal "assay reagents" for the study of a host of organic reactions essential for animal life.

Effect of Microbial Products Upon the Growth of Other, Notably Higher, Forms of Life. The microbiologist of today has made use of the vast potentialities of certain microbes for producing chemical substances that have the capacity to inhibit or stimulate the growth of other microbes, as well as of higher forms of life. Problems of saprophytism and parasitism are extending the ever-broadening horizons in microbiology and in life in general. The whole subject of chemotherapy has been revolutionized. Add to this the problem of animal nutrition that has gained considerably from our increasing knowledge of microbial products, preservation of food and other biological products, and human health and economy take on a new significance.

Numerous other aspects and approaches are characteristic of microbiology of today, and have tended to broaden the concepts of microbiology of yesterday. It is sufficient to mention the problems concerned with a better understanding of cellular morphology, of bacterial serotypes, of the toxin-antitoxin concept, and of quantitative immunology. The para-agglutination phenomena of yesterday have led to recognition of the serological reactions of the bacterial polysaccharides

and the transformation of bacteria by deoxyribonucleic acid of today.

Other phases of microbiology have recently made much progress. These include: (a) Problems in medical microbiology, such as the recognition of certain virulence factors and the creation of new types of vaccines. (b) The utilization of microbes in the study of their effects on living cells. (c) The potential use or misuse of microbes as a weapon of warfare.

Education in Microbiology. We must not, finally, overlook the problem of training of microbiologists. I can do no better than to quote, in this connection, B. C. J. G. Knight who, in speaking on this subject, stated "that the early microbiologists had a broad and integrated view of their subject, which was soon lost as their successors became preoccupied with practical applications. Microbiology should still be regarded as a single discipline, since all studies involving small and ubiquitous organisms use similar techniques and since most of the effects produced by such organisms are consequences of the same general property of multiplication.... The fundamental unity of biochemistry and physiology underlying the diverse activities of microorganisms should be emphasized and the biology and physiology of the different groups should be treated on a comparative basis."

In a generalized way the microbiologist of today is to be considered primarily as a specialist. He may be completely unaware of the broad biological aspects of the microbes responsible for a given reaction. He may even be accused of considering the microbe as a "bundle of enzymes" or as a "complex method of chemical reactions"; he may look upon microbes as primitive forms of life devoid of biological properties and possessing purely biochemical characteristics. He occasionally reminds one of the biochemist who, having worked all his life with the blood of a horseshoe crab without ever having seen one of these creatures, finally came upon one crawling on the main street in Woods Hole and was astounded by the queer nature of the animal. In fact, how many microbiologists of today have a microscope handy, to look at the organisms with which they are working No wonder the academic preparation for a microbiologist of today stresses a thorough training in chemistry and physics, and places little, if any, emphasis on botany, zoology, medicine, or agriculture, which provided the tools for the microbiologist of yesterday.

An Outlook.

While microbiology attained the stature as a science, it also became a tool for allied scientific fields and lost many of its demarcation lines. At the same time the contributions of most individual investigators have become diluted in team research. All this has led to a situation which permits us to distinguish in a generalized but significant way between the microbiology of today and of yesterday. Though we cannot draw a distinct line of demarcation between the microbiologist of yesterday and the microbiologist of today, it is apparent that they represent two different scientific entities. The new knowledge—we might even say the new philosophy or even sophistication—that characterizes the present-day microbiologist represents a totally different scientific approach to what may even appear to be a new problem. Perhaps we may be forgiven if in closing we raise the question whether, with all our modern scientific erudition and paraphernalia, modern contributions can equal in grandeur those made by the microbiologist of yesterday.

Possible remedies to narrow the gap of difference between the average investigators of today and yesterday may be suggested: Encourage delayed specialization of students, foster broad discussions straddling the remaining demarcation lines of scientific endeavors, place a premium on one or two postdoctoral years in a field different from that pursued during graduate years, and make courses in the history and philosophy of science obligatory, etc. Finally, by all means, discourage overwhelming project research and encourage long-term fundamental research programs. Give the individual investigator the opportunity to express himself freely, without stifling him by administrative duties, on the one hand, and the burden of supervision of numerous technicians, on the other.

BOOK REVIEWS

G. F. Gauze, Methods of Search for New Antibiotics (Moscow Acad. Sci. USSR Press, Popular Science Series, 1958) Edition of 5000 copies.

P. N. Kaskhin

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Gauze's monograph consists of a foreword, five chapters and a conclusion: a literature list is given for each chapter.

The introduction indicates the main aims of the monograph and notes that, besides information taken from the literature, it gives the results of research by the author and his colleagues in the Institute for the Search for New Antibiotics of the Academy of Medical Sciences, USSR.

The first chapter deals with the distribution of antibiotic-producing microbes in different soils. It gives information on the distribution of antibiotic-producing molds, sporeforming bacteria, and actinomycetes in various soils. The importance of geographical conditions for the distribution of antibiotic-producers is shown, and it is pointed out that, as a general rule, antagonistic microorganisms are of much more frequent occurrence in strains isolated from southern soils and the spectrum of their antibacterial action is broader.

From an analysis of the conducted research it is suggested that antibiotics in northern soils have a relatively greater protective action for the microbes producing them, possibly because of the greater stability of antibiotic molecules at the low temperatures of northern and alpine regions.

The same chapter contains the results of works of several authors on the formation of antibiotics in sterile and in nonsterile soil, and on the effect of the soil microflora on the formation of antibiotics and on their retention in the soil.

The second chapter discusses the classification of antibiotic-producing actinomycetes with reference to the search for new chemical substances. The greatest attention here is given to actinomycetes.

Taking concrete examples the author stresses the inadequacy of research on the systematics of actinomycetes and the unsatisfactory state of their classification. Of the modern schemes of classification of actinomycetes the principles of classification adopted by N. A. Krasil'nikov and S. A. Waksman are briefly described; the advantages of Krasil'nikov's classification are pointed out.

Some principles of the classification of actinomycetes are critically examined and schemes for their classification, based on experience acquired from work in the Institute of Antibiotics of the Academy of Medical Sciences, USSR, are given. Stress is laid on the importance of knowing the species of actinomycetes and the antibacterial spectrum of the antagonist for the discovery of producers of new chemical substances. However, the author does not deal at all with questions of the antigenic specificity of actinomycetes or with the importance of actinophages in the determination of actinomycetes.

The author uses the term "toxone". The definition of this term, however, is vague and requires to be made more precise.

The third chapter is devoted to the problem of the search for antibacterial antibiotics. Here there is a short account of the main stages in the search for antibiotics, and the necessary information is given on ways and methods of discovering antagonistic microbes, the following methods, in particular, being dealt with. 1) Soil enrichment, 2) bacterial agar plates, 3) congested population on agar media, 4) direct inoculation of soil. A comparative evaluation of some of the methods is given.

The author is perfectly correct in pointing out "the complexity of the phenomenon of antagonism and the need to employ the greatest variety of methods in order to discover all the various species and varieties of actinomycetes in any soil sample". From experience gained in the Institute for the Search for New Antibiotics the author cites the most suitable nutrient media for isolating actinomycetes from soil, and indicates the best medium for discovering the antibacterial antagonism of actinomycetes.

There is a brief account of the methods used to test the antagonistic properties of microorganisms on solid media and in culture fluids; the test microbes used for the first demonstration of antagonists are indicated and a scheme for the study of the antagonism towards various microbes is given. There is a short treatment of information relating to the cultivation of antibiotic-producers, the isolation of active substances from the culture fluid, and the testing of the chemotherapeutic properties of antibiotics. In concluding the chapter the author rightly stresses the importance of the search for new antibiotics, the necessity for combined work by different specialists and teams of scientists dealing with this problem.

Nevertheless, we cannot share the view expressed by the author that penicillin is in every case an extremely effective means for the treatment of infections caused by gram-positive bacteria. Owing to the wide spread of penicillin-resistant microorganisms, its efficacy and curative value decrease from year to year.

Nor can we share the author's views that oral administration of neomycin and colimycin results in "sterilization of the contents of the gastrointestinal tract" (p. 58). This is hardly likely from general considerations and, in particular, could hardly be without harm for the organism.

Finally, we cannot accept without question the author's assertions that clear zones round colonies of

antagonistic microbes are due to the dissolution of microbes susceptible to them (p. 61). The clear zones are due mainly to the bacteriostatic and bactericidal effect and only partially to the dissolution of microbes.

The fourth and largest chapter of the monograph is devoted to the problem of discovering antiviral antibiotics. It contains a brief account of methods of assaying antiviral action, and information is given on the antiviral action of products of the vital activity of molds, bacteria and actinomycetes with reference to the activity of several antiviral substances, such as helenine, ehrlichin, netropsin, cardicin, antivirusin, vascosin, cerulomycin, etc.

The author gives concrete illustrations of the various relationships between the activity of the same substances in vivo and in vitro, and notes the importance of the latter for the discovery of antiviral antibiotics.

Particular attention is given to the employment of bacteriophages in the search for antiviral antibiotics; there is an account of some properties of actinomycete-derived antibiotics which inhibit various bacteriophages (nibomycin, chrysomycin, phagolessin, alavin), and reference is made to their different nature, their different mode of action (phagocidal and phagostatic), and the various ways of isolating them (from mycelium, culture fluid).

There is a separate discussion of information on actinophages, their occurrence in nature, methods of isolating actinophages and counting their particles, as well as of the results obtained by the author and his colleagues, who have shown the varied selectivity of the lytic action of actinophages and the different sensitivity of various actinomycetes towards them.

There is a fuller account of the results of the research by the author and his colleagues on the specificity of actinophages, the different types of their interaction with actinomycete cultures, and on the inhibiting and stimulating effect of actinomycetes on actinophages. The observations were made on large numbers of actinomycetes (1000 cultures), isolated from soils from various geographical localities.

In concluding this chapter the author correctly stresses the view of N. A. Krasil'nikov and his colleagues to the effect that "antiviral action found with an actinophage model is not at all indicative of any universal antiviral action", and in view of this, substances inhibiting actinophages must be tested in relation to other viruses.

The use of actinophages alone can hardly be an efficient way of isolating specific antibiotics active against pathogenic viruses.

The fifth chapter deals briefly with some theoretical questions of the search for anticancer antibiotics, and reference is made to methods of testing anticancer activity and certain substances possessing the latter (actinomycin of S. A. Waksman, 1940).

Considerable space is devoted to cancer-cell "equivalents" in microbiology and their use in the search for anticancer antibiotics.

The author refers to work on obtaining malignant formations "cytologically very similar to animal tumors" in basidiomycetes, mushrooms and other multicellular and unicellular fungi.

Research was conducted involving the use of carcinogenic and other factors to obtain various mutant yeasts with the defective respiratory apparatus typical of cancer cells.

A fairly full account is given of the results of research by the author and other workers in the Institute for the Search for New Antibiotics of the Academy of Medical Sciences, USSR on methods of discovering mutants of true yeasts, Staphylococcus aureus, colon, and paracolon bacilli, and Bacillus mycoides, which possess a damaged respiratory apparatus. The mutants were obtained by the action of the ultraviolet irradiation and urethane. Common features of these mutants were the hereditarily stable reduction (up to 20–80%) in the respiratory quotient as compared with the original forms and various injuries to the cytochrome system, with a permanent shift of the cytochrome line.

The author finds features common to cancer cells in the modification of the respiratory function of the microbial variants and concludes that there is a similarity between cancer cells and microbial biochemical mutants with damaged oxidation.

In special observations conducted on 2500 cultures of different actinomycetes freshly isolated from soil samples of different origin, there were discovered 53 cultures (about 2%) which possessed a selective inhibitive action on staphylococcal biochemical mutants with damaged oxidation, but had no effect on the original normal cultures of staphylococci. Out of 53 cultures, ten suppressed mouse ascites tumor cells in vitro. From the observations made the author rightly notes: "It is difficult to say if substances selectively inhibiting the growth of microbial biochemical mutants with defective oxidation have any practical value in the treatment of malignant tumors". In fact, there is a need for further research on the specifity of the discovered analogy, for a comparative characterization of the viability of experimental mutants with defective oxidation, and for a study of the relation between their viability and their resistance to the action of antibiotic substances. The latter is particularly important for assessing the selective action of substances on microorganisms "equivalent" to cancer cells.

It must be regretted that the general account of mutants with defective oxidation gives no comparative data on the activity of their development, on their growth curves, or on their yield, these being important features in the characterization of cancer cells.

In the conclusion to the monograph the author again emphasizes the importance of the cooperation of microbiologists, biochemists and chemotherapists for the successful search for new antibiotics, not only against the agents of bacterial and viral infections, but also against cancer.

The book gives a short review of a whole series of works on the important theoretically interesting, and practically valuable problem of the search for antibiotics.

Special attention is given to antiviral antibiotics and anticancer remedies of microbial origin.

Critical comments are made on several works. There is a very full and illustrated account of the results of research by the author and his colleagues in the Insti-

tute of New Antibiotics, methods of further research on several problems are dealt with, and some interesting ideas are expressed.

As critical comment the following points appear appropriate:

1. The disproportionate amount of space given to the work of our own and foreign authors and to the results of his own observations. The results of research by the author and his colleagues in the Institute of New Antibiotics, already published in the periodical literature, are given in most detail and are better illustrated. An extremely brief treatment, sometimes a mere mention, is given to the studies of other authors, and some of our own scientists' works, read at congresses or printed in the proceedings of other institutes, are not mentioned by the author at all.

2. The rather brief review of the classification of actinomycetes cannot be considered as exhaustive. It is hardly worthwhile in one chapter of a short popular science monograph to analyze the principles and schemes of actinomycete classification, which is the subject of a very voluminous and controversial literature, requiring special analysis and, of course, a fuller account of the factual information.

3. The author does not devote sufficient attention to an important aspect of modern chemotherapy—the selection of preparations capable of suppressing the vital activity of disease-causing bacteria resistant to the action of already known antibiotics. The general biological significance of the increased resistance of microbes to antibiotics is not discussed, and the ways and methods employed in research on this problem are not touched on at all.

4. There is too brief reference, merely a mention, given to the use of ion-exchange resins for isolating antibiotics. There should have been a fuller account of this promising line of research. There is also only a brief mention of the use of antibiotics in stock-raising, poultry-farming, and the rearing of fur-bearing animals.

5. The book ought to have: a) Named the persons who introduced the various methods of isolating antagonistic microbes, particularly since the year of introduction of particular methods is given; b) defined virulence and pathogenicity, terms frequently used in the monograph; c) referred to the importance of experimental models of various infectious diseases in the search for new antibiotics.

6. Some terms employed by the author can hardly be considered as very happy choices: a) The "polychemism" of actinomycetes (p. 58); b) "local" and "celomic" preparations, when dealing with the use of preparations (p. 58); c) the "lytic region", from which pure cultures of actinomycetes are isolated (p. 64); d) naming as "new" antibiotic substances obtained 15–16 years ago, such as gramicidin (p. 62).

7. It would be more correct: a) To call fungi of the genus Candida yeastlike organisms, and not yeasts, since they do not have spores; b) not to use the term "number" instead of "quantity".

8. The monograph also contains some slips; not all the works cited by the author in the text are given in the literature index, and vice versa.

CONFERENCE ON MICROBIOLOGICAL METHODS OF OBTAINING FODDER PROTEINS

M. M. Makarova and V. Ya. Chastukhin

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The problem of utilizing fungi by culturing them on various industrial effluents (the wash of alcohol-hydrolysis and sulfite factories, the wash of continuous-process alcohol factories, etc.) was dealt with in the papers presented by the Department of Microbiology and Biochemistry of Leningrad Technological Institute.

V. Ya. Chastukhin reported new results on the ability of mycelial fungi, as distinct from yeasts, to utilize more fully the organic part of industrial effluents, and thus to contribute to the obtention of greater yields of fodder protein (betaine and other substances in molasses wash were in mind). In view of this the speaker recommended the development of research aimed at the discovery and cultivation of new species of microorganisms from natural sources, so that there would be a greater range of cultures most productive as regards the synthesis of protein, vitamins and biostimulators.

Goncherova's report was devoted to the chemical composition of mycelial fungi. Special attention was given in this paper to the results on the amino acid composition of mycelial fungi.

Several questions relating to the submerged culturing of mycelial fungi were discussed in detail in R. N. Golubchina's paper.

P. A. Yakimov dealt in his paper with the state and prospects of the production of semi-processed antibiotics and mycelial biomass from the utilization of the carbohydrate complex of the hydrolysis industry.

Participants in the discussion observed that the industrial obtention of fodder protein at present was based mainly on the fodder yeast industry. The productive resources, however, were still not being utilized sufficiently, and the fodder yeasts produced in Leningrad Economic Region were being directed to other regions of the country. At the same time, emphasis was laid on the great importance of extensive adoption of improved ensilage methods involving the use of specially selected bacterial starters, which ensure the better preservation of the proteins and vitamins in the silage obtained.

The participants at the conference noted the timeliness of its convening and the necessity to hold further conferences of specialists and scientific workers on these problems.

The resolutions of the conference advocated the need for increasing the production of fodder yeasts and starting cultures for ensilage, the extension of research into the selection of new microbial producers of proteins and biostimulators, and into the effect of microbial proteins and biostimulators on animals.

The conference noted that the USSR at present possessed no single leading research center coordinating work in this field; it was suggested that a petition should be placed before the government of the USSR advocating the creation of an Institute of Fodder Protein and Biostimulators in one of the centers where work in this field was already being carried out. In the opinion of many participants at the conference, Leningrad represented such a place.

A conference on the industrial production of fodder protein was convened jointly by the Leningrad Section of the All-Union Microbiological Society, Academy of Sciences, USSR, the Leningrad Regional Boards of the scientific and technical associations of the paper and wood-processing industry and the food industry, the Leningrad Council of National Economy, and the Leningrad Regional Board of Agriculture.

The conference was held in Leningrad during June 23–25, 1959.

Besides members of the above-named bodies, the conference was attended by workers in agriculture and in the food and hydrolysis industries, and also by representatives of several scientific research institutions. Altogether, 70 persons were present.

A. P. Dmitrochenko delivered a lengthy report on the question of employing yeasts and mold mycelium as supplementary sources of fodder protein. An account of the aims of the agricultural workers of Leningrad Region in the production of animal produce was given in a paper by N. A. Sheldybaev, the deputy chief of the Regional Board of Agriculture of the Leningrad Regional Executive Committee.

Considerable attention at the conference was given to the problem of the utilization and the technology of production, and to growth factors of microbial origin. Members of staff of the Institute of the Hydrolysis Industry read several papers on this question.

K. P. Andreev gave an account of the newest information on the technology of obtaining fodder yeasts, the prospects of employing continuous methods of fermentation, and the aims of scientific research in this field.

In a paper on the prospects of utilizing excess yeasts from the alcohol shops of hydrolysis and sulfite alcohol factories as fodder M. Ya. Kalyuzhnyi reported that from hydrolyzates and also from sulfite lyes, it was possible to take off up to 1 kg of pressed yeast per $1\,m^3$ of the processed medium without reducing the yield of alcohol from the fermenting sugar or prolonging the period of fermentation.

The economic aspect of fodder yeast production from hydrolyzates and ways of improving it were discussed in the papers of A. A. Andreev and A. I. Kozlov (Scientific Research Institute of Hydrolysis and Sulfite Alcohol Industry).

A. M. Malkov paid particular attention in his paper to methods of preparing and refining hydrolyzates for fermentation. However, in commenting on Malkov's paper A. P. Kryuchkova (Moscow Section of Scientific Research Institute of Hydrolysis and Sulfite Alcohol Industry) stated that the methods of refining hydrolyzates by treating them with iron salts and increased aeration, as recommended by Malkov, were not being used at present and no plans had been made for their introduction. The main problem at the moment did not lie in the purity of the hydrolyzate, but in the choice of the most stable and productive strains of fodder yeasts —the active producers of proteins and vitamins.

B. N. Gavrilov reported in his address on the effectiveness of employing supplementary yeast feeding in agriculture. These investigations, conducted under the guidance of T. V. Vinogradova, showed that supplementary feeding with yeast accelerated the development of the bees, increased the fecundity of the queen and the productivity of the bee colonies.

A detailed account of the state and prospects of fodder yeast production in Leningrad Economic Region was given in the report of A. M. Shiryaev (Leningrad Council of National Economy), and by F. A. Lukovnikov of the Byborg Central Design Bureau.

A separate session of the conference was devoted to biological methods of processing and preserving fodder.

M. M. Makarova (All-Union Scientific Research Institute of Agricultural Microbiology) gave an account in her paper of new results in the utilization of microbial producers of physiologically valuable substances for improving the quality of silage and the better preservation of proteins and vitamins in ensiled green fodder. She specially stressed the great significance of further research to discover new species of microorganisms and to select combined cultures of protein- and biostimulator-producers for the bacterial inoculation of silage prepared on farms. Great interest was evoked by the paper of A. E. Slukhai-Natal'chenko (All-Union Scientific Research Institute of Agricultural Microbiology) on the use of an Azotobacter culture in the feeding of farm animals. Experiments revealed that the feeding of suckling and weanling pigs with 10–15 ml per head of Azotobacter culture led to a gain in weight of 1–2.3 kg per month over that of control piglets.

F. I. Lyagushin (Dnepropetrovsk) reported on the efficacy of a paste of Azotobacter culture.

The question of enriching straw with proteins as a result of its partial hydrolysis by processing with water at pressure up to 4 atm, with subsequent fermentative treatment and cultivation of fodder yeast was the subject of a paper by N. S. Nikitina (All-Union Scientific Research Institute of Hydrolysis and Sulfite Alcohol Industry).

A. A. Golikova reported on a method developed in the All-Union Institute of Agriculture Microbiology for the enrichment of straw by treatment with a small dosage of lime and a combined starting culture consisting of acetic acid bacteria and yeasts, a method recommended by the institute also for the ensilage of fodders on the ground. The enrichment of straw and poor fodder hay by a complex of microorganisms consisting of lactic acid bacteria and yeasts, as observations showed, provides a means of maintaining the yield of a dairy herd when the farm experiences difficulty with its fodder supply.

THE SEVENTIETH BIRTHDAY OF S. A. WAKSMAN

A. A. Imshenetskii

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In 1959 the Institute of Microbiology of Rutgers University published a collection of papers read at a session commemorating the birthday of Prof. S. A. Waksman. The name of S. Waksman is widely known to microbiologists throughout the world as that of an outstanding scientist who has enriched microbiology with works of fundamental importance and who discovered streptomycin.

S. Waksman was born on July 22, 1888 in the town of Priluka near Kiev. In 1910, when he had finished the fifth Odessa gymnasium, he emigrated to the U.S.A. He entered Rutgers College in 1911 and finished in 1915 with the degree of Bachelor of Agriculture. This was followed by work on soil microbiology at the New Jersey Agricultural Experiment Station as an assistant under Prof. Lipman. In 1916 Waksman was awarded the degree of Master of Science, and two years later he received the degree of Doctor of Science in Biochemistry at California University. When he returned to Rutgers he occupied the post of Microbiologist at the Experiment station, and at the same time filled the post of Lecturer in Soil Microbiology at the college.

In 1925 Waksman became an associate professor, and in 1930 head of a department. In 1940 a Department of Microbiology was set up in the University and its first head was Prof. Waksman. In 1949 the Board of Rutgers University organized a new specialist Institute of Microbiology and appointed Prof. Waksman as its director, a post which he held until 1958. He continues to work actively, particularly as an author of books, with the energy so characteristic of him.

In 1931 Prof. Waksman was invited to organize a marine microbiology section at Woods Hole Oceanographic Institution. He led this section till 1942.

At various times during his career Prof. Waksman was a scientific adviser on questions of microbiology for several state institutions, particularly the Department of Agriculature, as well as an adviser to various scientific research institutes and industrial undertakings.

Waksman is a member of scientific societies of many countries, including France, Sweden, Mexico, India, Brazil, etc. He is a member of the American National Academy of Sciences and the American Academy of Arts and Sciences, a foreign member of the French Academy of Sciences, and has been elected President of the Society of American Bacteriologists, President of the Soil Microbiology Committee of the International Society of Soil Science and Vice-President of the American Society of Agronomy.

For his outstanding investigations in the field of microbiology Prof. Waksman has been awarded the degree of Doctor of Sciences honoris causa by the following universities and colleges: Liege, Strasbourg, Jerusalem, Athens, Rutgers, Princeton, Pennsylvania, Philadelphia, Janonville, New York, Keio and others.

Over a period of thirty years Prof. Waksman has received numerous awards and medals: in 1947—the Emil Christian Hansen Award of the Carlsberg Institute in Denmark, in 1950—the Leeuwenhoek Medal of the Dutch Academy of Sciences, in 1948—a medallion of the New Jersey Agricultural Society, an award of the American Academy of Arts and Sciences, the Albert and Mary Lasker Award of the American Public Health Association and many more prizes awarded by various scientific associations, academies of sciences and clubs.

In 1952 the scientist was awarded a Nobel Prize for Physiology and Medicine. Prof. Waksman has also received a Legion of Honor and the Order of Merit of the Rising Sun.

The scientific work of Prof. Waksman can be divided into two periods. From 1916 to 1940 he worked in the field of biochemistry, soil and marine microbiology. His studies on the biochemistry of soil microorganisms were particularly numerous. His investigations dealt with almost all sections of soil microbiology—decomposition of vegetable and animal remains in the soil by microbes, formation and nature of soil humus, the microbial population of different soils, the oxidation of sulfur by bacteria, the biology of microbes involved in the circulation of nitrogen and carbon in soil. During this period Waksman published the following monographs and manuals: Enzymes (1926), Principles of Soil Microbiology (1927, 1932), Soil and Microbes (1932), Humus (1936, 1938) and Soil Microbiology (1952). During this period Prof. Waksman, enjoying a very high reputation and endowed with exceptional erudition, was one of the leading soil microbiologists in the world.

From 1940 the trend of Prof. Waksman's work changed. He became interested in the problem of the antagonistic interrelationships of microbes. He studied the formation and nature of antibiotic substances, primarily those produced by actinomycetes. This led to intensive research on the systematics, morphology, physiology, biochemistry, distribution and methods of culturing various actinomycetes. Waksman himself, as well as in cooperation with his colleagues, discovered several new antibiotics: actinomycin (1940), clavacin, streptothricin (1942), streptomycin (1943), grisein (1946), neomycin (1948), fradicin, candicidin, etc. Of these, streptomycin and neomycin have received general recognition and are widely employed.

From his prolonged study of the antagonistic properties of microbes Prof. Waksman was able to write and have published several monographs on this question. These included Microbial Antagonisms and

Antibiotic Substances (1945, 1947), Actinomycetes (1950), Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics (1953), as well as collections published under his editorship Streptomycin – Nature and Practical Applications (1949), Literature on Streptomycin (1948, 1952), and Neomycin (1952, 1956).

As already mentioned, in 1949 the Board of Rutgers University decided to set up an Institute of Microbiology at the University. It should be mentioned that the Institute was constructed and equipped largely from the funds accruing as royalties from the success of streptomycin and neomycin. Part of this money was also used to found a special fund to render assistance to the microbiological research being conducted in various countries.

Owing to the high prestige and energetic leadership of Prof. Waksman the Institute soon became a first-class scientific research organization, which acquired a well-merited reputation as a scientific center, primarily on the problem of antibiotics. We need only peruse the published reports of the work of the Institute to see how comprehensive is the research work undertaken there and what highly qualified scientific personnel are being trained and are involved in the work of the Institute. The Institute undertakes the training of specialists, which later defend their doctoral dissertations or, if they already have a doctorate, study for higher qualifications.

Prof. Waksman has had more than 400 works published. Most of them are devoted to the results of experimental studies, but besides these he has written several articles on the history of microbiology, as well as works of a popular-scientific or autobiographic character. These include the excellent monographs Sergei N. Winogradsky and My Life with the Microbes. At the meeting held in the Institute of Microbiology of Rutgers University to celebrate Prof. Waksman's 70th birthday many microbiologists, scientists and friends of the guest of honor were present. The first paper was given by Prof. Quastel, a biochemist working in Montreal (Canada), on the theme "Microbial biochemistry and its development". In a brief account of the significance of the studies and discoveries of Buchner, Harden, and Young, Meyerhof, Neuberg, Ehrlich, Abderhalden, Raistrick, Winogradsky, Beijerinck, Omelyanskii, etc. Prof. Quastel pointed out that after the work of these scientists over a period of thirty years, all the attention was concentrated on the diversity and complexity of processes taking place in the cell and associated with the production of energy and the synthetic processes necessary for the growth and multiplication of microbial cells. The approach to the microbial cell as a dynamic biochemical system has led to an understanding of many previously obscure aspects of the metabolism of microbes. After this, the speaker discussed current information on energy metabolism, the mechanism of adaptation, assimilation processes, the synthesis of polysaccharides, the synthesis of nucleic acids and protein, and the action of antibiotics. In concluding, Prof. Quastel pointed out that microbial biochemistry is just as extensive as biochemistry itself, and that in any field of science new techniques of study provide new results, and new theories and ideas lead to new discoveries.

The second paper was that of Woodruff, Director of the Microbiology Laboratory of Merck and Co., on the theme "Antibiotics—a new field for microbiological research and perspectives for the future". In this paper considerable attention was devoted to the chemical structure of antibiotics, and to their action on certain viruses.

A paper entitled "Episodes in immunochemistry" was read by Heidelberger, Professor of Immunochemistry in Rutgers Institute of Microbiology. Cowan, the Curator of the National Collection of Type Cultures in London, delivered a paper on "Bacterial classification —problems and developments", which was of interest both to general and medical microbiologists. The speaker was right in stressing that the erection of a phylogenetic classification was a complex problem and that the investigator had relatively little factual information for this purpose. He later discussed the taxonomic importance of morphological features, relationship to stains, habitats, serological data, pathogenicity, nutrient requirements, nature of substances synthesized, genetical and other information. On examining the problem in relation to the resistance of species and the trend of future research on bacterial systematics, the speaker pointed to the existence of two schemes of classification. One of them owed its origin to the independent fields of microbiology—medical, veterinary, food microbiology, etc.—which erected their own practical classification required in the particular field. In addition to this there existed a general system, which claimed, though not always with adequate basis, the designation "natural" or "phylogenetic". This paper on bacterial systematics, despite several controversial viewpoints, contained interesting ideas and, what was particularly valuable, excellent illustrations of the whole complexity of bacterial systematics. Prof. Bryson of Rutgers Institute of Microbiology read a paper on the theme "Some contributions of genetics to microbiology". This paper was interesting in that it gave a review of the earlier studies on the variability of microbes, particularly those of Beijerinck, Gurney-Dickson, as well as the works of Waksman on variability in the actinomycetes. Of interest also were the references to the little-known works of Henry, who obtained anthrax bacillus mutants by means of ultraviolet radiation in 1914, i.e., 14 years prior to Muller's corresponding work with Drosophila.

In this paper a great deal of attention was given to an account of spontaneous and induced mutations, as well as to a comparison of the past and present achievements in microbial genetics.

A paper on the theme "Aspects of Russian microbiology" was read by Skryabin (Institute of Microbiology, Academy of Sciences, USSR). He gave a short historical account of the development of microbiology in prerevolutionary Russia and a review of the achievements of Soviet microbiology.

In place of concluding remarks the guest of honor delivered a paper which bore the same title as the collection of papers as a whole, namely "Microbiology

yesterday and today". In this paper he analyzed the different stages of development of microbiology and showed how the former, purely biological trend of research on microbes gradually changed to physiological and biochemical research. This analysis and assessment of the future of the various lines of research could only have been given by a great scientist who could clearly foresee the future of microbiology, a scientist who possessed a lifetime of experience, profound knowledge and great insight.*

Prof. Waksman's seventieth birthday finds him full of constructive ideas and still working very actively. He is writing a three volume work on actinomycetes, the first volume of which has already been published, and he still takes part in the work of international congresses and symposiums.

Prof. Waksman is not only an eminent research worker, a great scholar and an outstanding specialist in microbiology. His thorough study of the problem of antagonism was crowned with the discovery of streptomycin, the value of which can hardly be overestimated. We all know how the treatment of tuberculosis victims has been facilitated by the discovery of streptomycin, and the people of all countries must be grateful for such a brilliant contribution to the treatment of serious infections. To know that the results of one's studies have directly reduced the mortality from such an ailment as tuberculosis is the greatest happiness for a scientist.

Soviet microbiologists have always had a very high regard for Prof. Waksman as a scientist and have always been glad to see him during his several visits to their native country. They sincerely wish health to Prof. Waksman and hope that he will retain for many years that boundless energy which is so characteristic of all his scientific activity.

*A translation of this paper is printed in this issue of the journal.

F. M. CHISTYAKOV

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On October 15, 1959, Professor Fedor Maksimovich Chistyakov, Doctor of Technical Sciences, Head of the Food Commodities Department of the G. V. Plekhanov Moscow Order of the Labor Red Banner Institute of National Economy, passed away.

F. M. Chistyakov was born into a peasant family in 1898. In 1914 he began his working life as a village teacher. In 1920 he joined the ranks of the Communist Party of the Soviet Union, and was an active member till the end of his life. As a member of a partisan brigade during the Civil War he took part in the rout of Kolchak, after which he assisted in the organization of Soviet bodies in the provinces, and up until his last days Fedor Maksimovich carried out great social and political work on the instructions of the Party. In 1929, after finishing at the G. M. Plekhanov Moscow Institute of National Economy, Fedor Maksimovich was offered postgraduate training in the Institute's microbiological laboratory, which was headed by Prof. Ya. Nikitinskii (junior). The work conducted by Fedor Maksimovich on acetone-butanol fermentation was of great practical value, and for this work he was awarded the degree of Candidate of Technical Sciences.

From 1932 Fedor Maksimovich carried out important scientific teaching and administrative work, holding in various years the post of director of teaching and research in several scientific research institutes and colleges (All-Union Research Refrigeration Institute, Moscow Institute of Soviet Cooperative Trade, Lvov Institute of Trade and Economics, G. V. Plekhanov Moscow Institute of National Economy).

F. M. Chistyakov was an eminent specialist in the field of food microbiology and the science of commodities. He had about 40 scientific works published on questions of microbiology, processing and preservation of food products. His works are characterized not only by the depth of theoretical research, but also by their close link with practice, with the demands of industry.

F. M. Chistyakov was a pioneer in research on the microbiology of the cold storage of food products. In 1945 he defended a dissertation for the degree of Doctor of Technical Sciences on the theme "The effect of low temperatures on microorganisms in relation to the cold storage of products". F. M. Chistyakov was a member of the committee of the International Institute of Refrigeration. His papers on questions of the effect of cold on microorganisms were read at international congresses held in 1955 (in Paris) and in 1959 (Moscow). F. M. Chistyakov was the first in our country (in the thirties) to devise a method of microbiological control of preserves production.

Up to the last days of his life Fedor Maksimovich carried out fruitful work in the training of scientific personnel and the preparation of young specialists for the national economy. He was a highly qualified teacher. His lectures were distinguished by the newness of his material and depth of content.

Fedor Maksimovich was the author of several textbooks and manuals on food microbiology.

From 1940 to 1947 Fedor Maksimovich was a member of the editorial board of the Journal "Microbiology". For his valuable work Fedor Maksimovich Chistyakov was awarded two orders and five medals of the Soviet Union.

A vivid memory of Fedor Maksimovich will long remain with those who knew him.

A. N. Vyshelesskii, M. S. Zabolotskii, V. V. Eremenko, A. A. Imshenetskii, N. I. Kozin, V. V. Kozlov, S. I. Ledovskikh, D. I. Lobanov, K. A. Mudretsova, A. S. Razumov, and Ya. I. Rautenshtein.

THIS ISSUE OF MICROBIOLOGY CONTAINED A SUPPLEMENTARY (PAGES I to VI) ARTICLE COMMEMORATING THE 90TH ANNIVERSARY OF THE BIRTH OF V. I. LENIN. THIS ARTICLE HAS NOT BEEN TRANSLATED INASMUCH AS IT DOES NOT DEAL WITH EITHER HISTORICAL DETAILS OF THE PROGRESS OF MICROBIOLOGY IN THE SOVIET UNION OR WITH CONTEMPORARY ASPECTS OF SOVIET RESEARCH IN MICROBIOLOGY.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Eksptl. Biol. i Med.	Byulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

* BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

SIGNIFICANCE OF ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN SOVIET PERIODICALS

FIAN	Phys. Inst. Acad. Sci. USSR.
GDI	Water Power Inst.
GITI	State Sci.-Tech. Press
GITTL	State Tech. and Theor. Lit. Press
GONTI	State United Sci.-Tech. Press
Gosenergoizdat	State Power Press
Goskhimizdat	State Chem. Press
GOST	All-Union State Standard
GTTI	State Tech. and Theor. Lit. Press
IL	Foreign Lit. Press
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd. AN SSSR	Acad. Sci. USSR Press
Izd. MGU	Moscow State Univ. Press
LEIIZhT	Leningrad Power Inst. of Railroad Engineering
LET	Leningrad Elec. Engr. School
LETI	Leningrad Electrotechnical Inst.
LETIIZhT	Leningrad Electrical Engineering Research Inst. of Railroad Engr.
Mashgiz	State Sci.-Tech. Press for Machine Construction Lit.
MEP	Ministry of Electrical Industry
MES	Ministry of Electrical Power Plants
MESEP	Ministry of Electrical Power Plants and the Electrical Industry
MGU	Moscow State Univ.
MKhTI	Moscow Inst. Chem. Tech.
MOPI	Moscow Regional Pedagogical Inst.
MSP	Ministry of Industrial Construction
NII ZVUKSZAPIOI	Scientific Research Inst. of Sound Recording
NIKFI	Sci. Inst. of Modern Motion Picture Photography
ONTI	United Sci.-Tech. Press
OTI	Division of Technical Information
OTN	Div. Tech. Sci.
Stroiizdat	Construction Press
TOE	Association of Power Engineers
TsKTI	Central Research Inst. for Boilers and Turbines
TsNIEL	Central Scientific Research Elec. Engr. Lab.
TsNIEL-MES	Central Scientific Research Elec. Engr. Lab.–Ministry of Electric Power Plants
TsVTI	Central Office of Economic Information
UF	Ural Branch
VIESKh	All-Union Inst. of Rural Elec. Power Stations
VNIIM	All-Union Scientific Research Inst. of Meteorology
VNIIZhDT	All-Union Scientific Research Inst. of Railroad Engineering
VTI	All-Union Thermotech. Inst.
VZEI	All-Union Power Correspondence Inst.

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. – Publisher.

AIBS Russian Monograph Translations

The AIBS is in the process of expanding its Russian Translations Program extensively. Funds to subsidize translation and publication of important Russian literature in biology have been obtained from the National Science Foundation, as part of a larger program to encourage the exchange of scientific information between the two countries. The following monographs have been published:

Origins of Angiospermous Plants. *By A. L. Takhtajan.*
Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.
68 pp. $3.00, individuals and industrial libraries (U.S.A. and Canada);
$2.00, AIBS members and all other libraries; $.50, additional, foreign.

Essays on the Evolutionary Morphology of Plants. *By A. L. Takhtajan.*
Edited by G. Ledyard Stebbins. Translated by Olga H. Gankin.
144 pp. $5.00, individuals and industrial libraries (U.S.A. and Canada);
$4.00, AIBS members and all other libraries; $.50, additional, foreign.

Problems in the Classification of Antagonists of Actinomycetes. *By G. F. Gause.*
Edited by David Gottlieb. Translated by Fritz Danga.
$5.00, individuals and industrial libraries (U.S.A. and Canada);
$4.00, AIBS members and all other libraries; $.50, additional, foreign.

Arachnida. Vol. IV, No. 2. Fauna of the U.S.S.R. *By B. I. Pomerantzev.*
Edited by George Anastos. Translated by Alena Elbl.
$10.00, individuals and industrial libraries (U.S.A. and Canada);
$9.00, AIBS members and all other libraries; $1.00, additional, foreign.

Arachnoidea. Vol. VI, No. 1. Fauna of the U.S.S.R. *By A. A. Zachvatkin.*
Translated and edited by A. Ratcliffe and A. M. Hughes.
$10.00, individuals and industrial libraries (U.S.A. and Canada);
$9.00, AIBS members and all other libraries; $1.00, additional, foreign.

Marine Biology. Vol. XX, Trudy Institute of Oceanology. *Edited by B. N. Nikitin.*
$10.00, individuals and industrial libraries (U.S.A. and Canada);
$7.50, AIBS members and all other libraries; $1.00, additional, foreign.

Plants and X-rays. *By L. P. Breslavets.*
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The American Institute of Biological Sciences, under a grant from the National Science Foundation, is currently translating and publishing the following seven Russian research journals in biology.

DOKLADY

BIOLOGICAL SCIENCES SECTIONS

This is a translation of all papers in the Biological Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections in many areas of biology, among which are the following: biophysics, cytology, embryology, experimental morphology, genetics, histology, hydrobiology, microbiology, morphology, physiology, and zoology.

6 issues per year. First issues published: 1957, Vols. 112-117. Current issues published: 1960, Vols. 130-135.

YEARLY SUBSCRIPTIONS: $20.00, individuals and industrial libraries (U.S. & Can.); $15.00, AIBS members and all other libraries; $2.50 additional to each price, foreign. SINGLE COPIES: $4.00.

DOKLADY

BOTANICAL SCIENCES SECTIONS

This journal is a translation of all papers in the Botanical Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections on botany, plant anatomy, plant embryology, plant morphology, and plant physiology.

6 issues per year. First issues published: 1957, Vols. 112-117. Current issues published: 1960, Vols. 130-135.

YEARLY SUBSCRIPTIONS: $7.50, individuals and industrial libraries (U.S. & Can.); $5.75, AIBS members and all other libraries; $1.50 additional to each price, foreign. SINGLE COPIES: $1.50.

DOKLADY

BIOCHEMISTRY SECTION

This is a translation of all papers in the Biochemistry Section of the Proceedings of the Academy of Sciences of the USSR. These papers deal with all phases of the chemistry of plant and animal life, at both the biological and physiological levels. This translation contains a list of abbreviations most frequently encountered in Russian Bio-sciences literature, as well as a reference list of Russian journals that are frequently cited.

6 issues per year. First issues published: 1957, Vols. 112-117. Current issues published: 1960, Vols. 130-135.

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PLANT PHYSIOLOGY

(FIZIOLOGIYA RASTENII)

This journal publishes original studies of an experimental nature on various problems of the physiology of plants, reviews, critical articles, and discussions of major problems in this field. It has special sections dealing with methods of experimental research and the application of finished scientific research to current agricultural practice.

6 issues per year. First issues published: 1957, Vol. 4, Nos. 1-6. Current issues published: 1960, Vol. 7, Nos. 1-6.

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MICROBIOLOGY

(MIKROBIOLOGIYA)

This journal deals with problems of general microbiology (systematics, morphology, physiology, and variability of microorganisms), agricultural microbiology (microbiology of soils and fodder crops, bacterial fertilizers), technical microbiology, the microbiology of foods, and the microbiology of water reservoirs. This journal also contains sections on the history of science, book reviews, and current events in the field of microbiology, as well as the general articles in this field.

6 issues per year. First issues published: 1957, Vol. 26, Nos. 1-6. Current issues published: 1960, Vol. 29, Nos. 1-6.

YEARLY SUBSCRIPTIONS: $20.00, individuals and industrial libraries (U.S. & Can.); $15.00, AIBS members and all other libraries; $2.50 additional to each price, foreign. SINGLE COPIES: $4.00.

ENTOMOLOGICAL REVIEW

(ENTOMOLOGICHESKOE OBOZRENIE)

Very few entomologists would not find something of special interest to them in any one issue of this periodical. It deals with theoretical and practical problems in the field of entomology and their significance for the national economy. It contains articles on all branches of entomology and devotes particular attention to problems connected with the theoretical basis of agriculture and public health service.

4 issues per year. First issues published: 1958, Vol. 37, Nos. 1-4. Current issues published: 1960, Vol. 39, Nos. 1-4..

YEARLY SUBSCRIPTIONS: $25.00, individuals and industrial libraries (U.S. & Can.); $12.00, AIBS members and all other libraries; $3.00 additional to each price, foreign. SINGLE COPIES: $7.50.

SOVIET SOIL SCIENCE

(POCHVOVEDENIYE)

This is a translation of the Russian journal, Pochvovedeniye, edited by Dr. A.P. Mazurak of the University of Nebraska. It publishes research done at various institutes in the fields of soil chemistry, soil physics, soil microbiology, soil fertility, soil genesis and classification, soil conservation, irrigation, and related aspects of soil science.

12 issues per year. First issues published: 1958. Current issues published: Jan.-Dec. 1959.

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